

T cells in HBV associated hepatocellular carcinoma and other cancers



Xi Li
College: Christ Church

Supervisors:
Professor. Tao Dong
Professor. Sir. Andrew McMichael

2017

Thesis submitted to Nuffield Department of Medicine,
University of Oxford for the degree of Doctor of Philosophy
in Clinical Medicine

Acknowledgements

Firstly, I sincerely express my grateful thanks to all the patients who were involved in studies in this thesis. In total, samples from over 200 patients were collected for this research. Most of them were suffering the pains of cancer. Some of them were confronting terminal illness and yet kindly donated the surgical samples or peripheral blood for our research. Without their altruism, this study could not have been completed.

Throughout my D Phil, studies, I am grateful for the role of my supervisor, Prof. Tao Dong, who introduced medical sciences to me and gave me the opportunity to conduct a summer internship project in my final year, as an intern doctor in the medical school in 2011. Since then, I have been determined to pursue a D Phil study in clinical medicine using this valuable experience. In 2012, I was privileged to be able to join her lab and start my D Phil in a clinically relevant project, characterizing T-cells exhaustion in HBV infection and cancers, under her supervision. In the 4 years of study, my knowledge of medical research has been broadened, which strengthened my understanding of the diseases in depth. I believe I will benefit from this training in my clinical career for the rest of my life.

I am immensely grateful to my co-supervisor, Professor. Sir. Andrew McMichael for his guidance and teaching in T cells immunity of HIV, antigen specific T cells, enthusiastic support and encouragement as well as all the useful discussions through each stage of my D Phil study.

I would also express my thanks to all the colleagues in the 2 collaborative hospitals in China. I especially thank Prof. Ruozhen Wang from Xinjiang Cancer Hospital for her strong support in this collaborative project as well as her wonderful coordination and hospitality. Her efforts resulted in obtaining high quality surgical samples and precise clinical information for our study. I express thanks to Dr. Yunhui Hu and all team members in Prof. Wang's group for their coordination and help. In parallel, I thank Dr. Yonghong Zhang, members in Zhang's lab, Dr. Dongdong Lin, Dr. Libo Song and Dr. Haitao Zhang in Beijing You'an hospital for all their support and help in obtaining surgical samples and conducting experiments. Particularly I am thankful to Prof. Shichun Lu for in China Military General Hospital for his supports and efforts in patients' recruitment. I deliver special thanks to Lin Qin for all the crucial help and support as well as her accompaniment of endless optimization in my D Phil study.

I thank all the postdocs and group members in Prof. Dong's lab for suggestions and help they have kindly given me. In addition, the banter and gossip about me enjoyed by the female postdocs, made my life in Oxford colourful. I am thankful for the help and fun from Dr. Yanchun Peng, Chi Zhang, Dr. Sophie Andrews and Henry Laurenson-Schafer in the lab. I deliver my special thanks to Dr. Xuan Yao for her efforts in helping me process tissues and coordinate experiments in both collaborative hospitals in China. I appreciated the help from Xijin Xu in bioinformatics and programming languages. Thanks to Prof. Seph Borrow, Prof.

Sarah Rowland-Jones in the NDM research building as they have provided useful suggestions and thought-provoking discussion with me regarding my project.

I am grateful to WIMM FACS facultie's, Paul Sopp and Craig Waugh for all the critical help in flow cytometry. I specially thank Paul for his efforts in solving Fortessa's problems (in China, remotely) and in training visitors from both collaborative hospitals.

I express my sincere thanks to Bill Radford as he proof read some of my academic works and provided useful suggestions in academic writing.

Finally, I would thank my uncle who guided me into medicine and illuminated the beauty of surgery, the combination of art and science, for me. I shall also express great thanks to my mom in loving what I was doing and supporting me unconditionally. I hope I could make her proud. The same thanks go to my grandparents for all the caring and support you have given to me.

Abstract

Individuals with HBV associated hepatocellular carcinoma or other cancers are characterized by dysfunctional anti-virus and anti-tumor T-cell responses. Since noteworthy benefits of inhibitory pathway blockades, such as PD-1 inhibitor or CTLA-4 inhibitor have been achieved in multiple advanced cancers, the next critical question is which mono- or combinatorial blockade regimens may reinvigorate anti-virus and anti-tumor T-cell immunity in multiple cancers. This thesis aims to comprehensively characterize the expression patterns of 8 known inhibitory receptors on T cells in order to provide a rationale for the design of next generation blockade immunotherapy to benefit to more individuals with advanced cancers.

This thesis has shown that PD-1 and Tim-3 are significantly up-regulated on both CD4 and CD8 TILs when comparing to its expression on PBMCs in 19 paired PBMCs-TILs HBV associated HCC and 39 breast cancer individuals. This indicates PD-1 and Tim-3 blockade may be a promising target in HBV associated HCC and breast cancer immunotherapy. Furthermore, the combinatorial expression analysis of the co-expression of PD-1, CTLA-4, 2B4, KLRG-1 and TIGIT identifies 2B4+KLRG-1+TIGIT+PD-1-CTLA-4- as the dominant phenotype of CD8 TILs in HBV associated HCC; 2B4+KLRG-1-TIGIT+PD-1+CTLA-4-and as the shared dominant phenotypes of CD8 TILs in breast cancer, gastric cancer, esophageal cancer, colorectal cancer, lung cancer and renal cancer individuals. Our data indicates that the combinatorial blockade of additional pathways such as 2B4, TIGIT and KLRG-1 along with PD-1 pathway may increase the overall response rate in multiple cancer immunotherapy. Furthermore, this thesis also identified the positive correlation between PD-1 expression on TILs and PD-L1 expression in tumors from non-triple negative breast cancer individuals. It indicates that PD-L1 may have the value as a biomarker in selecting patients who may benefit from PD-1 pathway blockade in non-triple negative breast cancer individuals.

In addition, this thesis also applied multiple bioinformatics algorithms to identify the distinctive expression of inhibitory receptors on T-and NK-cells in PBMCs from a cross-sectional chronic HBV cohort (N=66). The hierarchical clusters in the CITRUS algorithm showed down-regulation of 2B4 on the CD3+CD8+CD56+ subset in HBV-associated cirrhosis individuals when compared with chronically infected individuals and healthy donors. In parallel, the CITRUS algorithm also identified reduced co-expression of 2B4 and TIGIT on the CD3-CD8+CD56+ subset in HBV-associated cirrhosis and HBV-associated HCC when compared with healthy donors. The mono- or combinatorial down-regulation of 2B4 and TIGIT may indicate the activation of NKT cells and NK cells respectively in the late stages HBV-associated disease (cirrhosis and HCC subgroups), which may have an important role in HBV-associated disease progression, although more functional studies are necessary to confirm this finding.

Presentations and award resulting from the work in this thesis

20th October 2013: Oral presentation in HBV International Meeting entitled “T cells in HBV related hepatocellular carcinoma”

3rd October 2014: 3rd Oral Presentation Award, entitled “T cells in HBV associated HCC”, HIU Day, Weatherall Institute of Molecular Medicine, University of Oxford

27th March 2015: Poster presentation in WIMM Day Weatherall Institute of Molecular Medicine, University of Oxford

8th April 2016: Poster presentation in WIMM Day, Weatherall Institute of Molecular Medicine, University of Oxford

1 CHAPTER 1: INTRODUCTION.....	9
1.1 Epidemiology of HBV chronic infection and HBV associated HCC	9
1.2 Hepatitis B virus biology and genotypes	11
1.2.1 HBV molecular virology	11
1.2.2 HBV genotypes.....	13
1.3 Clinical courses of HBV infection	14
1.3.1 HBV acute infection.....	14
1.3.2 HBV chronic infection	15
1.3.2.1 Immune-tolerant phase of chronic HBV infection	16
1.3.2.2 Immune-active phase of chronic HBV infection.....	16
1.3.2.3 Inactive carrier of HBV chronic infection	17
1.3.2.4 Reactivation phase of HBV chronic infection	17
1.4 Inhibitory pathway blockades in chronic infection and cancer treatments	17
1.5 PD-1 inhibitory pathway.....	22
1.5.1 Discovery of PD-1 and its ligands	22
1.5.2 PD-1 pathway in tumor microenvironment.....	23
1.5.3 Anti PD-1/PD-L1 pathway immunotherapy in the clinic	26
1.6 CTLA-4 inhibitory pathway	27
1.6.1 Discovery of CTLA-4 and its function.....	28
1.6.2 Anti-CTLA-4 in the clinic.....	29
1.7 Tim-3 inhibitory pathway	30
1.7.1 Expression of Tim-3 and its ligands.....	30
1.7.2 Tim-3 pathway in diseases	31
1.8 KLRG-1 inhibitory pathway	33
1.8.1 Expression of KLRG-1 and its ligands	33
1.8.2 KLRG-1 inhibitory pathway and its role in cancer	34
1.9 2B4 pathway	35
1.9.1 Expression of 2B4 and its ligand	35
1.9.2 Dual roles of 2B4.....	36
1.10 TIGIT inhibitory pathway	37
1.10.1 Expression of TIGIT and its ligands.....	37
1.10.2 TIGIT inhibitory pathway in chronic virus infection and cancer	38
1.11 CD160 and BTLA inhibitory pathways	39
1.11.1 Discovery of CD160 and BTLA and their ligand	39
1.11.2 CD160 and BTLA pathways in cancer	40
1.12 Molecular pathways of inhibitory receptors in T cells	41
Hypotheses to test and aims of this thesis:.....	44
2 CHAPTER 2: MATERIAL AND METHODS	46
2.1 Study cohort and healthy donors.....	46
2.1.1 Beijing HBV associated HCC cohort, China	46
2.1.2 Xinjiang cancer cohort, China	46
2.1.3 Oxford healthy donors cohort, UK.....	47
2.1.4 Inclusive and exclusive criteria of surgical samples from multiple cancer individuals....	47

2.2 Peripheral blood mononuclear cell separation.....	49
2.3 Optimization of intrahepatic lymphocytes isolation	49
2.4 Mechanical and enzymatic dissociation of intrahepatic lymphocytes.....	52
2.4.1. Isolation of intrahepatic lymphocytes	52
2.4.1.1 Mechanical Isolation of intrahepatic lymphocytes.....	53
2.4.1.2 Enzymatic Isolation of intrahepatic lymphocytes.....	54
2.3 Human tumor dissociation and immune cells separation	55
2.4 Multichromatic flow cytometry panels.....	58
2.4.1 Panel design for the analysis of inhibitory receptors expression on T cells and T-cell differentiation.....	58
2.4.2 Applying FMO control in the gating of inhibitory receptors in multichromatic flow cytometry	62
2.5 Paraffin embedded human tissues treatment and staining	67
2.5.1 De-waxing of paraffin embedded sections.....	67
2.5.2 Antigen retrieval of paraffin embedded sections	67
2.5.3 Treatment for autofluorescence reduction in paraffin embedded human tissues	69
2.5.4 Fluorescent staining in human paraffin embedded sections.....	70
2.6 Quantification of ligands expression by confocal microscopy.....	71
2.7 Data analysis.....	72
2.7.1 Data analysis of the expression of inhibitory receptors on T cells.....	72
2.7.2 Data analysis of correlation between the expressions of inhibitory receptor and ligand	72
2.7.3 Big data analysis in FIJI software with R language.....	72
2.7.4 High-throughput bioinformatics in single cell analysis	73
2.7.4.1 SPADE analysis.....	73
2.7.4.2 viSNE analysis.....	73
2.7.4.3 CITRUS analysis	74
3 CHAPTER 3: EX VIVO CHARACTERIZATION OF INHIBITORY RECEPTORS ON T CELLS FROM DIFFERENT CANCER INDIVIDUALS	75
3.1 Introduction	75
3.2 Results.....	77
3.2.1 Characterization of sole inhibitory receptor expression on T cells between peripheral blood and tissues.....	77
3.2.2 Variable sole inhibitory receptor expression on tumor infiltrating T cells from different cancer individuals.....	93
3.2.3 Less variable sole inhibitory receptor expression on circulating T cells from different cancer individuals.....	102
3.2.4 Stage based <i>ex vivo</i> characterization of sole expression of inhibitory receptors on T cells from breast cancer individuals	106
3.2.5 Identification of combinatorial expression of inhibitory receptors on CD8+ tumor infiltrating T cells from different cancer individuals.....	112
3.2.5 Implementing “immunoscores” of CD8+ TILs to dissect T cells from different cancer environments.....	123
3.2.6 Comparative analysis of T-cell differentiation between peripheral blood and tissues...	127
3.2.7 Expression analysis of inhibitory receptors in different T-cell differentiation stages between the peripheral and cancer tissues.....	130
3. 3 Discussion	136

4 CHAPTER 4: DISSECTING LIGANDS EXPRESSION FROM DIFFERENT TUMOR MICROENVIRONMENTS	141
4.1 Introduction	141
4.2 Results.....	144
4.2.1 Optimization of the paraffin embedded human sample treatments for ligand expression analysis by confocal microscopy	144
4.2.2 Differential ligands expression pattern in tumor microenvironment.....	149
4.2.2.1 PD-L1 has membranous, cytoplasmic expression patterns in breast cancer tumor microenvironment.....	150
4.2.2.2 e-cadherin has membranous expression pattern on breast cancer tumor microenvironment.....	152
4.2.2.3 Galectin-9 has membranous pattern on breast cancer tumor microenvironment .	153
4.2.3 Identical hierarchical expression pattern observed between inhibitory receptors on tumor infiltrating T cells and its ligands expression in tumor microenvironment.....	156
4.2.4 Correlation between PD-1/PD-L1 was observed in paired breast cancer.....	164
4.3 Discussion	170
 5 CHAPTER 5: CHARACTERIZING T-CELL EXHAUSTION IN HBV CHRONICALLY INFECTED LIVER DISEASES	173
5.1 Introduction	173
5.2 Results.....	174
5.2.1 No differential expression of inhibitory receptors on peripheral T cells were observed among different stages of HBV chronically infected individuals.....	174
5.2.2 Applying bioinformatics analysis-CITRUS to reveal the heterogeneity of lymphocytes among different stages of HBV chronically infected individuals.....	180
5.2.2.1 CITRUS plots reveal the markers expression in differential lymphocytic subsets..	182
5.2.2.2 CITRUS analysis predicts subsets that exhibit significant phenotypes in different disease courses.....	194
5.2.2.3 Validation of subsets predicted by CITRUS that may demonstrate differential expression among different experimental groups	203
5.2.3 Applying SPADE algorithms for the expression analysis at individual level	209
5.2.4 Visualizing high dimensional flow cytometric data to single cell level by viSNE algorithms	220
5.2.5 Discussion	241
 6: FINAL DISCUSSION AND FUTURE DIRECTION	245
6.1 Multiple roles of inhibitory receptors.....	245
6.2 Tailored regimens for blockade immunotherapies	247
6.3 Identification of biomarkers for blockade immunotherapies	249
6.4 Adaptive resistance from blockade immunotherapies	250
6.5 Future plans	252
 7 APPENDIX:	253
 8 REFERENCES:	267

1 Chapter 1: Introduction

1.1 Epidemiology of HBV chronic infection and HBV associated HCC

Hepatitis B virus (HBV) currently infects over 350 million people worldwide, resulting in severe health threats to these patients and about 1/3 of the disease burden is in China[1]. Endemic areas of HBV include China, South-East Asia and Sub-Saharan Africa. However, Western Europe and the United States have a relatively low incidence of HBV infection. Vertical HBV transmission from mother to neonates dominates in Saharan Africa areas whereas, horizontal transmission via close personal contact or sexual contact is more common in China and South-East Asia especially in the areas where hygiene is not satisfactory. A prevalence of global chronic HBV infection map in 2014 is illustrated below in figure 1-1.

Figure 1-1 Global prevalence of chronic HBV infection in 2014

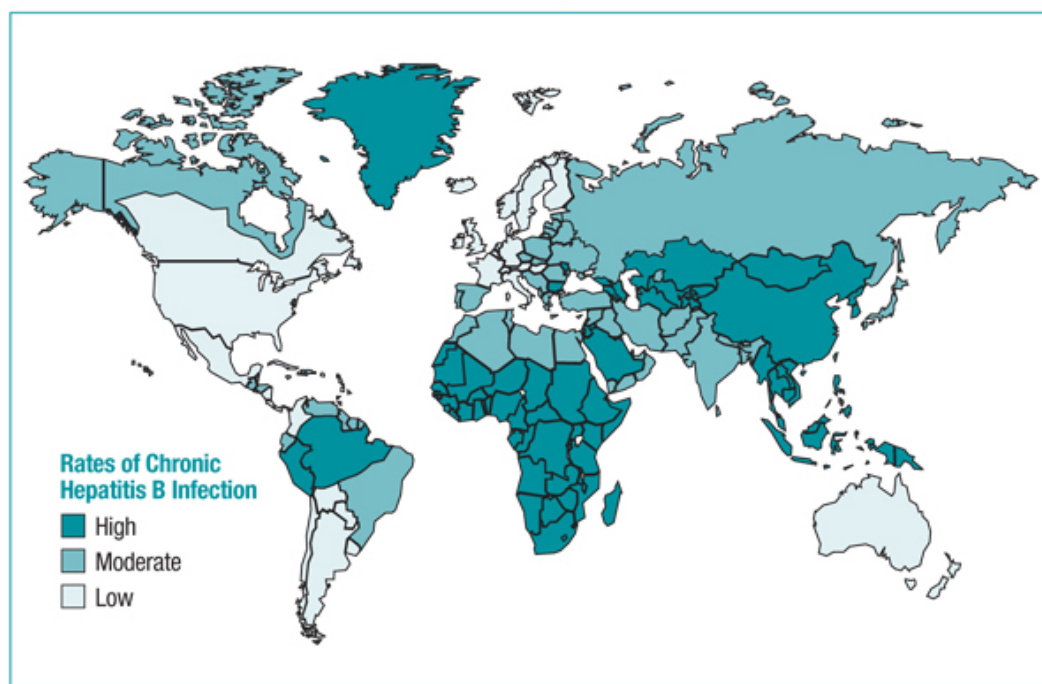


Figure was adapted and modified from Center for Disease Control website

The primary infections are mostly found in healthy adults, whether symptomatic or not, and are normally self-limited, with efficient anti-reinfection immunity and the clearance of the virus from both the liver and peripheral blood.

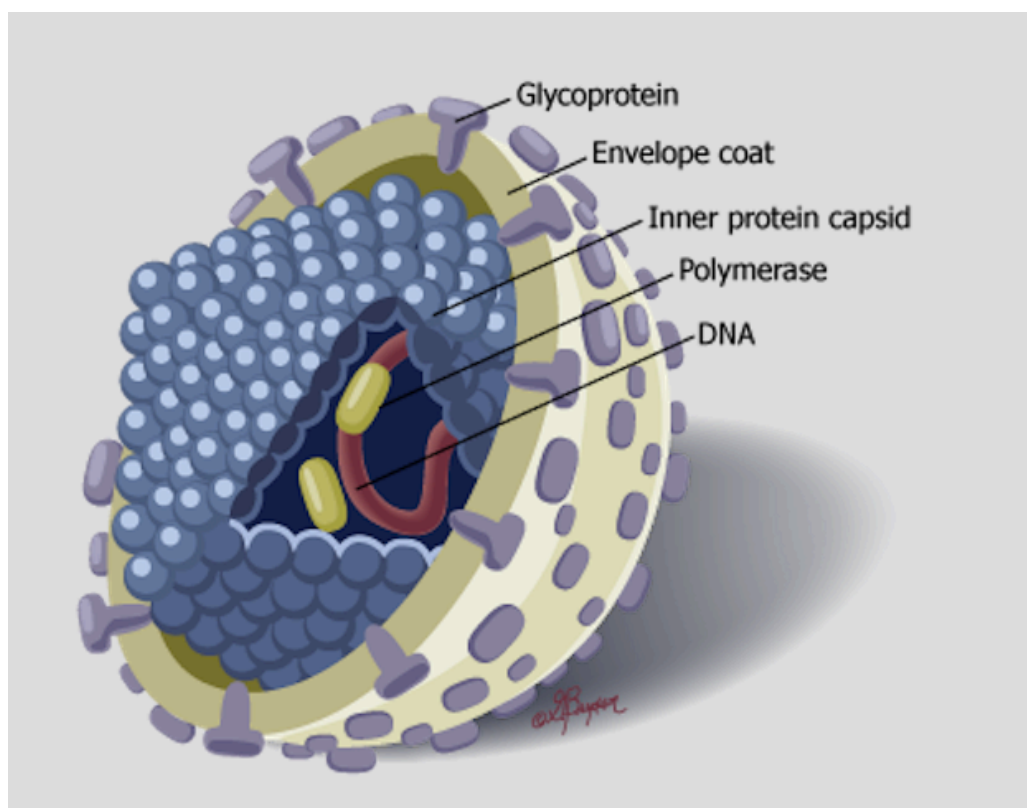
However, about 5% of primary infection patients will develop a persistent infection with or without abnormal liver functions. Overall, chronic HBV carriers have a 20- to 100-fold greater risk of developing hepatocellular carcinoma (HCC) compared to the uninfected general population, though other factors (*e.g.*, age, gender, exposure to aflatoxin, high consumption of alcohol, and co-infection with HCV) also carry various risks of HCC[2]. As the third most common cancer death-related disease, HCC is the fifth most common cancer in men and seventh in women [3, 4]. Each year, more than half million people in the world are diagnosed with HCC worldwide. About 50% of HCC cases are chronic HBV infected patients and this rate is much higher in children especially in areas such as East Asia and Africa where vertical transmission is not effectively controlled. About 90% percent of vertically transmitted HBV cases develop into chronic infection with high frequency of viral integration into host genomes[4]. In chronic HBV infections, after 20-30 years, about 20% to 30% of patients will develop liver cirrhosis. At an annual rate about 1%-7%, chronic HBV infected patients with liver cirrhosis develop to HCC[5].

1.2 Hepatitis B virus biology and genotypes

1.2.1 HBV molecular virology

Hepatitis B virus is a double-stranded circular DNA virus, about 40 to 42 nm in diameter with an outer lipoprotein envelope that contains three surface antigens[6]. Inside the envelope is the viral core that contains the viral genome, a partially duplex DNA of 3.2 kb, and a polymerase[7]. The HBV virus has four open reading frames, encoding several proteins including the pre-core and core proteins, the polymerase protein, small, medium and large envelope proteins and the X-protein[8]. 3D cartoon structure of HBV virion is illustrated in figure 1-2.

Figure 1-2: HBV virion structure



Cartoon of HBV virion adapted from Toronto center for liver disease website

The preS-S open reading frame (ORF) encodes 3 viral envelope proteins (viral antigens), Pre-S1 (large protein), Pre-S2 (medium protein) and S (small protein) respectively. Large protein has been thought to play a crucial role in host cell recognition and release of virion from infected cells[9, 10]. The function of Medium protein is still unknown. While the Small protein is the most abundant protein released in the blood also known as HBsAg, it is an important clinical marker for HBV chronic infection diagnosis.

The preC-C ORF encodes hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg). HBeAg is a non-structural protein and plays no role in viral assembly and is not a prerequisite for virus replication. HBeAg's function is not yet clear but it may involve in the induction of tolerance, shifting host immune responses to an immunotolerant Th2 profile[11, 12]. HBcAg consists the inner core protein shell known as core particle or nucleocapsid[8]. The presence or absence of HBeAg and HBcAg enable clinicians to define different stages of HBV chronic infection, which will be discussed in the following session.

The P ORF encodes polymerase, which is a multifunctional enzyme involving HBV DNA synthesis and RNA encapsidation[5]. The X ORF encodes HBx protein. HBx could have an impact on the host and viral gene expression and is thought to induce carcinogenesis, although it is not essential for viral replication[13, 14].

1.2.2 HBV genotypes

To date, the DNA sequences of HBV virus from patients' blood revealed at least 8 genotypes with differential geographic distribution (table 1-1 demonstrated detailed distribution of each HBV genotype)[15]. Genotype A is predominately pandemic in North America, North Europe, India and Africa; Genotype B and C are mainly found in Asia; Genotype D dominates in South Europe, Middle East and India; Genotype E is mainly disseminated in Africa; Genotype F, G and H are mainly in the American continent[16-20].

Table 1-1: HBV genotype distribution

<i>Genotype</i>	<i>Geographic Distribution</i>
A	Africa, India, Northern Europe, United States
B	Asia, United States
C	Asia, United States
D	India, Middle East, Southern Europe, United States
E	West and South Africa
F	Central and South America
G	Europe, United States
H	Central and South America, California in United S

Adapted and modified from Calvin Q. Pan et al; Int. J. Med. Sci. 2005 2(1)

In China where our cohort was established, the dominated HBV genotypes are B and C. The overall prevalence of HBV genotype B and genotype C in China is 65% and 33%[21]. HBV genotype B and genotype C account for more than 95% of HBV chronically infected individuals in China[22].

Within genotype C, the prevalence of HBV sub-genotypes is 42% in HBV sub-genotype C1 and 58% in HBV sub-genotype C2 in China[16, 22, 23]. In addition, HBV sub-genotype C1 is mainly detected in South China cities[24]. However, In Beijing and other Northern cities where our patients come from, the dominant sub-genotype is C2[24, 25].

1.3 Clinical courses of HBV infection

1.3.1 HBV acute infection

Upon the exposure to the HBV virus, individuals will develop immune responses to control the virus and establish immunity against reinfection. HBsAg becomes detectable in the circulation after an incubation period of 4 to 10 weeks[26]. With the detection of HBsAg, it is normally accompanied by high titer HBV viremia-frequently 10^9 to 10^{10} virions per milliliter[27]. Later antibodies against the HBV core antigen (anti-HBc antibodies) will be detectable. About 90% of adults can control HBV replication by eliciting potent immune responses. As a result, the HBsAg in peripheral blood could disappear with the appearance of Anti-HBs antibody. However, about 5% of adults could not successfully control HBV virus and then develop a persistent HBV infection with the consistent appearance of HBsAg in the blood for more than 6 months[8, 14].

1.3.2 HBV chronic infection

As opposed to self-limited acute HBV infection, when HBsAg persistently appeared in the blood for more than 6 months, a chronic HBV infection is established. Comparison of serologic markers and HBV DNA copies between acute HBV infection and chronic HBV infection is depicted in figure 1-3. Based on the serologic markers, chronically HBV infected individuals can be divided into 4 phases: immune-tolerant phase, immune active phase, inactive hepatitis B phase and reactivation phase[28].

Figure 1-3: Comparison between acute HBV infection and chronic HBV infection

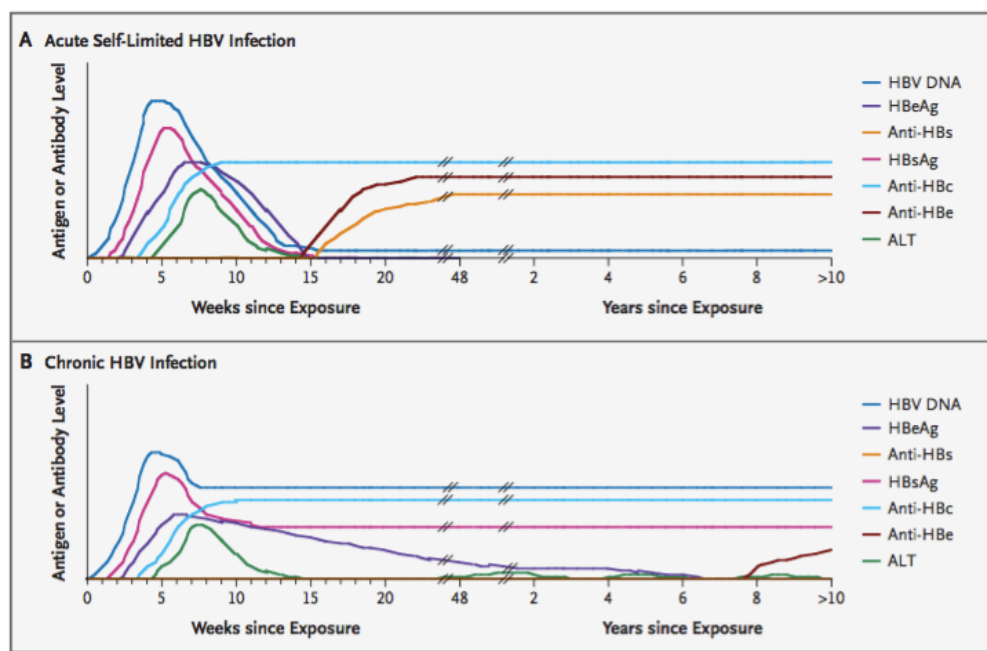


Figure was adapted from Don Ganem et al; n engl j med 350; 11, 2004

Figure 1-3: A: Upon primary infection, dynamic fluctuation of ALT, HBV DNA copies, HBsAg, HBeAg, anti- HBc, anti-HBe, and anti-HBs antibodies are shown in acute HBV infection (Figure1-3-A) and in chronic HBV infection (Figure 1-3-B).

1.3.2.1 Immune-tolerant phase of chronic HBV infection

HBV chronically infected individuals in this phase are characterized by high HBV viral load, presence of HBeAg, high level of serum HBsAg in the peripheral blood and normal or minimally elevated ALT. Age is a very important factor in this phase. If individuals infected by HBV vertically from HBeAg positive mother, this phase may last few decades[5]. HBeAg may contribute to the immune tolerance, which facilitates the virus avoiding immune surveillance. If individuals acquired HBV infection in the childhood, this phase could be shortened to 5-10 years. However, this phase may be absent or only last about 2-4 weeks in adult chronically infected individuals[15].

1.3.2.2 Immune-active phase of chronic HBV infection

The transition from immunotolerance to immunoactive phase of chronic HBV infection usually starts at the age between the ages of 20 to 40. But some patients may start earlier. The mechanism that triggers the “switch” of immune system from immunotolerance to immunoactive mediating responses towards virus and cause liver inflammation or damage is still unknown. In this phase, chronically HBV infected individuals are characterized by elevated ALT and liver damage, decline of HBV DNA copies and seroconversion of HBeAg positive to HBeAg negative/anti-HBe positive. This is a consequence of the immune system starting to recognize HBV as foreign antigen and mediate responses to the infected hepatocytes, causing liver inflammation and tissue damage with or without liver

fibrosis.

1.3.2.3 Inactive carrier of HBV chronic infection

The inactive carrier phase of chronically HBV infected individuals are characterized by the presence of anti-HBe and absence of HBeAg, low HBV DNA copies (less than 2000IU/ml), normal ALT and attenuated liver inflammation.

In this phase, it has been shown the HBV virus specific CD8 T cells are detectable in the peripheral blood although it has low frequency with distinctive exhausted phenotype[29].

1.3.2.4 Reactivation phase of HBV chronic infection

After the seroconversion of HBeAg, a certain proportion of HBV chronically infected individuals undergo spontaneous reactivation of HBV replication with the appearance of high HBV viral load (commonly over 20000IU/ml) and elevated ALT level. Only small percentage of this group of patients will regain serum HBeAg, the vast majority of reactivation patients are HBeAg negative/antiHBe positive. This may associate with the HBV virus Pre-core or core promoter mutations that interfere the production of HBeAg.

1.4 Inhibitory pathway blockades in chronic infection and cancer treatments

Physiologically, the homeostasis of co-stimulatory receptors and co-inhibitory receptors on T cells is finely tuned for the maintenance of self-tolerance to

prevent overreaction of immune responses and protecting tissues from immunomediated damages, while effectively eliciting potent responses when encountering pathogenic infection. However, if this dynamic counter-balance between co-stimulatory receptors and co-inhibitory receptors is disrupted, it can lead T cells to a dysfunctional state known as T-cell exhaustion. T-cell exhaustion has 2 significant characteristics; 1: T cells are functionally inactivated with poor cytokine production; 2: T cells are highly apoptotic with activation of multiple apoptosis signaling, resulting in CD8⁺ antigen specific T cells depletion.

In HBV chronic infection, intrinsic and extrinsic defects together contribute to T-cell exhaustion of intrahepatic lymphocytes. Intrinsic defects include the up-regulation and activation of BCL2-interacting mediator (BIM), a pro-apoptotic molecule that initiates HBV specific CD8⁺ T cell depletion. Intrinsic defect of T-cell exhaustion in HBV chronic infection is the up-regulation of multiple inhibitory receptors on both HBV specific and non-specific T cells. On the other hand, the extrinsic defects of HBV chronic infection include the unique liver environment consisting sinusoidal space lined of endothelial cells and other subsets, which nurtures multiple immunosuppressive cytokines such as IL-10, TGF-beta. In return, such an extrinsic factor attenuates intrahepatic T cells to expand and survive in the battlefield, the liver, of controlling HBV virus.

In tumor microenvironments, cancer cells are originated from somatic mutations, which produce antigenic proteins that are recognizable in the process of immune surveillance. In addition, in some solid cancers such as liver cancer, the oncofetal genes expression or cancer/testis proteins were up-regulated, which constitute tumor associated antigens (TAAs) that could be recognized by

CD8⁺ tumor infiltrating T cells and mediate defensive responses. Oncofetal TAAs consist of oncofetal alpha proteins, a very useful biomarker in clinical diagnosis of liver cancer and glypican-3 (GPC-3), an additional proposed biomarker in the diagnosis of solid tumors[30-33]. Cancer/testis TAAs include New York Esophageal Squamous Cell carcinoma 1 (NY-ESO-1), melanoma-associated antigen-1 (MAGE-A), synovial sarcoma X breakpoints 2 (SSX-2) and telomerase reverse transcriptase (hTERT)[34-37]. Those cancer/testis TAAs are overexpressed in multiple solid tumors with exceptional immunogenicity and prevalent prognosis[37-41]. However, despite the immunogenic capacity of TAAs, tumor microenvironment generates numerous immunosuppressive influences to evade immunoediting, one of which is to up-regulate the inhibitory receptors on tumor infiltrating T cells resulting in T-cells exhaustion. Several studies indicate that the TAA specific CD8⁺ tumor infiltrating T cells co-upregulated multiple inhibitory receptors such as PD-1, CTLA-4, Tim-3, OX40, KLRG-1 and 2B4[29, 42]. Specific co-expression patterns are suggested to associate with poor prognosis[43-46]. In 2011, the first inhibitory pathway inhibitor Ipilimumab, a monoclonal antibody blocking CTLA-4, was approved by the US Food and Drug Administration (FDA), which officially opens the new era of immunotherapy-inhibitory pathways blockade[47]. Since then, not only CTLA-4 but also PD-1 inhibitors, Nivolumab and Pembrolizumab have achieved promising results in treating metastatic melanoma individuals, non-small cell lung cancer individuals, advanced gastric cancer individuals and advanced bladder cancer individuals[48]. In the results of PD-1/PD-L1 inhibitors and anti-CTLA-4 inhibitor treatments from multiple clinical trials, differential Objective Response Rates (ORR) have been observed. For instance, the Nivolumab has

obtained consistent ORR of 30%-40% patients with advanced melanoma from multiple clinical trials[49, 50]. Meanwhile, Pembrolizumab has achieved ORR of 19% in the treatment of non small cell lung cancer individuals, an ORR of 20% in treating advanced gastric cancer individuals and an ORR above 20% in treating head and neck cancer individuals[51-53]. Currently PD-1 inhibitors have also been extended to the treatment of triple negative breast cancer (Estrogen receptor-, Progesterone receptor-, and Human epidermal receptor 2-), a subtype of poor prognosis breast cancer, individuals and classical Hodgkin's lymphoma individuals[54, 55]. Ever though noteworthy results have been obtained from multiple advanced cancer treatments, the blockade of inhibitory pathways revolutionizes the cancer treatment concepts and enhances our comprehension of cancer immunity. Current outstanding questions would be 1: extending its application to other advanced cancers; 2: investigating the immunological characteristics of non-responders under current treatment of PD-1 or CTLA-4 inhibitors; 3: developing additional inhibitory pathway blockades to achieve benefits for advanced cancer individuals; 4: characterizing combinatorial blockades of multiple inhibitory pathways to improve ORR and sustaining potent anti-tumor responses. Work in this thesis tackles these questions in a cohort comprising multiple primary cancer patients in collaborative hospitals in China. Apart from PD-1 CTLA-4inhibitory signaling, additional inhibitory pathways include KLRG-1, CD160, Tim-3, 2B4 TIGT, and BTLA that demonstrate prospective capabilities in blockade immunotherapies (Figure 1-4).

Figure 1-4 Inhibitory receptors and stimulatory receptors on T cells and NK cells

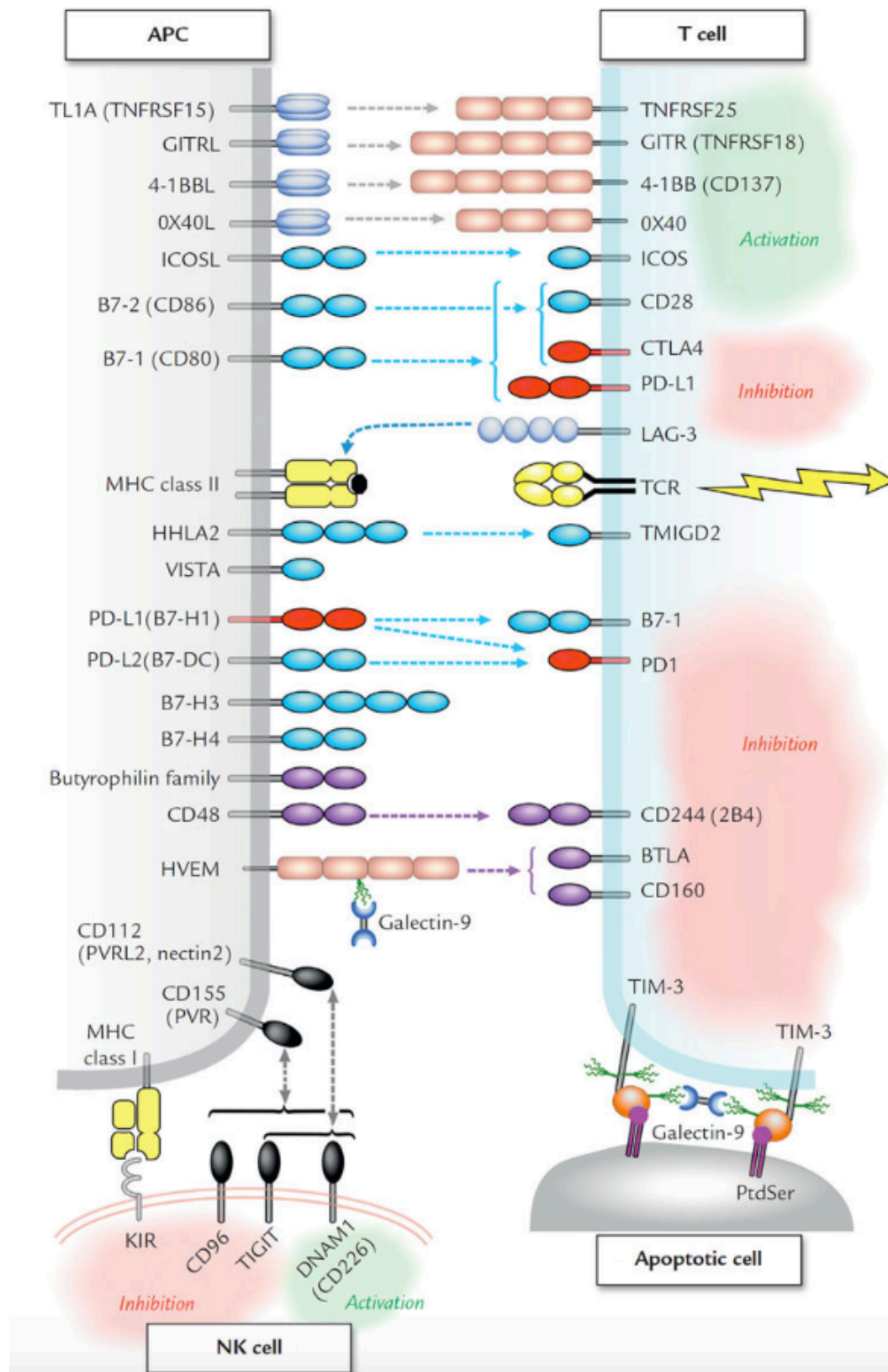


Figure was adapted from Kathleen et al; *Clin Ther.* 2015 April 1; 37(4)

Of note, some of them are not only the inhibitory receptors expressed on T cells but they also eliciting inhibitory function on other effector cell subsets such as NK cells. Each above-mentioned inhibitory receptor and its ligands will be discussed in detail in the following paragraphs.

1.5 PD-1 inhibitory pathway

1.5.1 Discovery of PD-1 and its ligands

PD-1 is a type I, 50-55kDa transmembrane glycoprotein with an single Ig-V type extracellular domain. Its sequences share 21-33% identity with CD28, CTLA-4 and ICOS[56]. Dating back to 1991, cDNA of PD-1 was firstly isolated from studies on 2 cell lines, being LyD9, a murine hematopoietic progenitor cell line undergoing programmed cell death with the activation of IL-3 and 2B4.11, a murine T-cell hybridoma cell line undergoing apoptosis with stimulation of phorbol myristate acetate and ionomycin[57].

Furthermore, several studies contributed to the discovery of one of PD-1's ligands, Program Death Ligand-1 (PD-L1) in 1999, which was initially named B7 Homolog-1, (B7-H1). Based on its homology of B7 family, using molecular cloning techniques and searches of the human expressed sequence database, it was demonstrated that PD-L1 serves as an inhibitory function of T cell responses in vitro[58]. Subsequently 2 studies indicated that PD-L1, as a binding ligand interacts with PD-1 negatively mediating T-cells activation[59]. Next, PD-L1 knock-out mice exhibited susceptibility to autoimmune diseases even though it was not developed spontaneously[60]. Apart from PD-L1, another ligand of PD-1, Program Death Ligand-2 (PD-L2) was identified in 2001 in 2 laboratories[61, 62]. It was named B7-DC as it predominately expressed on

dendritic cells and binds with PD-1 to elicit inhibitory function of T cells. Apart from binding with PD-1, both PD-L1 and PD-L2 could also interact with other molecules. PD-L1 is capable of binding CD80 on activated T cells mediating inhibitory signaling[63, 64]. In the meanwhile, PD-L2 has been found to bind to repulsive guidance molecule family member b (RGMb) which is highly expressed in lung macrophages that may involve self-tolerance[65]. Figure 1-5 illustrates the interaction of PD-L1 or PD-L2 with other molecules.

Figure 1-5: Currently known interaction of program death pathway

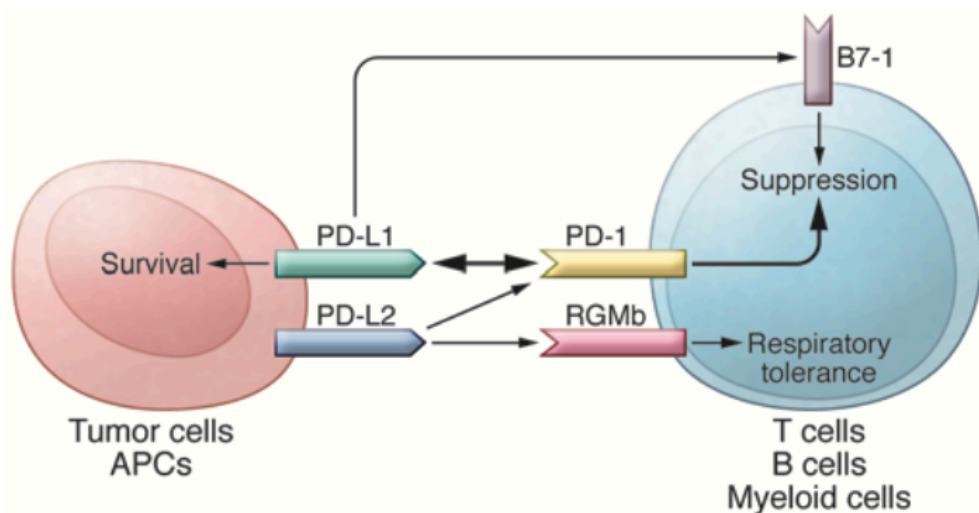


Figure was adapted from LP Chen et al; J Clin Invest. 2015;125(9)

1.5.2 PD-1 pathway in tumor microenvironment

PD-1 has been found to express on double negative α/β T cell and γ/δ T cells in normal thymus and induced on peripheral T cells and B cells upon activation[66, 67]. *ex vivo* phenotypic analysis also indicated that in benign liver tissues without chronic HBV/HCV infection, PD-1 is upregulated when comparing with its expression in the periphery[68]. In malignant tissues, a number of studies

have revealed that PD-1 expression is significantly up-regulated on tumor infiltrating T cells in patients from melanoma, liver cancer, gastric cancer, kidney cancer, non-small cell lung cancer, etc[56, 69, 70].

Generally, in most of the normal tissues, PD-L1 has very low expression. But in different organs it has differential expression. It has relatively high expression in liver tissues, lung tissues, placenta tissues but low expression in spleen lymph nodes and thymus. Whereas, PD-L2 is rarely detected in most of the normal organ tissues apart from lung[56].

Figure 1-6: Tumor adaptive resistance model

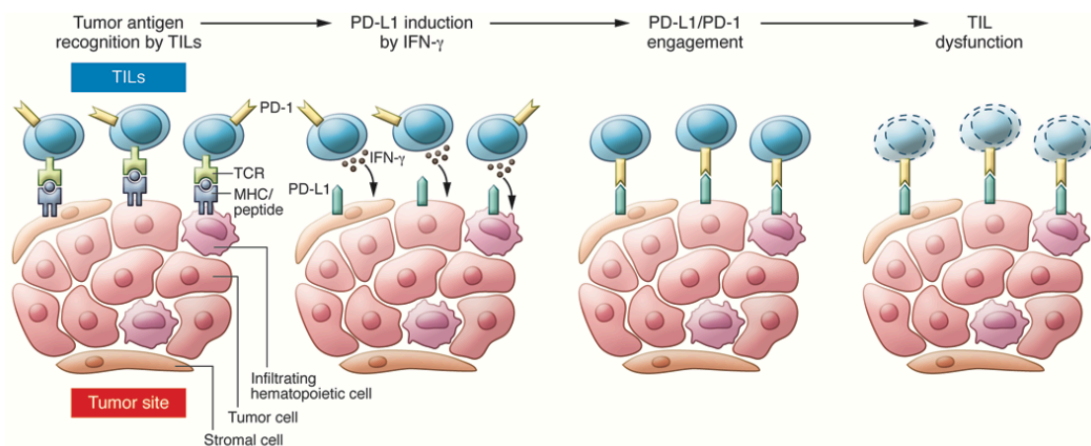


Figure 1-6 was adapted from LP Chen et al; J Clin Invest. 2015;125(9)

However, PD-L1 has been found to be significantly upregulated in malignant tissues from multiple cancers. Currently the most widely accepted theory about PD-1/PD-L1 axis is “adaptive resistance hypothesis” (figure 1-6) in which it is hypothesized that upon recognition of TCR with MHC-I molecules, tumor infiltrating CD8 T cells release IFN-gamma which then induce the expression of

PD-L1 on tumor cells and other antigen presenting cells such as macrophages and dendritic cells in tumor microenvironment serving as the binding ligand to PD-1 (figure 1-7). Then it negatively regulates the production of IFN-gamma. It is possible that the physiological function of the negative feedback by up-regulating PD-L1 is to limit the propagation of inflammation and tissue damage. This hypothesis is supported by the observation through immunohistochemistry studies in tumor tissues that PD-L1+ cells are only clustered adjacent to T cells[48]. In addition, strong positive correlation was observed between PD-L1 expression and aggregation of TILs in malignant lesions from melanoma patients[48]. However, despite the IFN-gamma dependent mechanism, PD-L1 may also express in an IFN-gamma independent manner. This is supported by the evidence that in a small proportion of human cancer tumor tissues, it has high level of PD-L1 expression with the absence of TILs[71, 72].

Figure 1-7: PD-1/PD-L1 pathway induced immunosuppression in tumor microenvironment

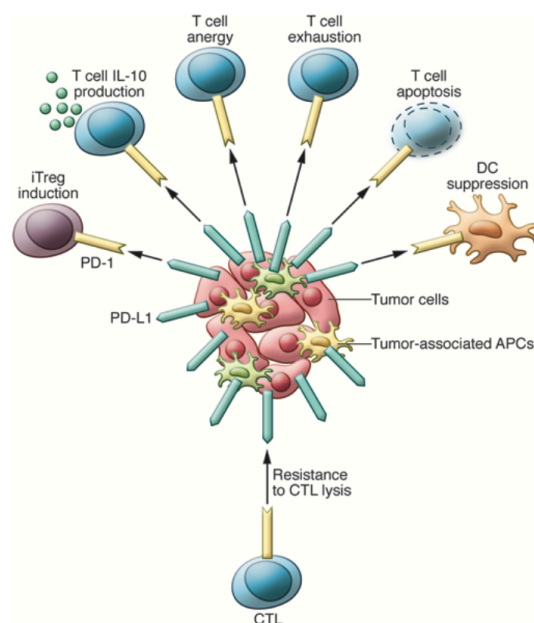


Figure 1-7 was adapted from LP Chen et al; J Clin Invest. 2015;125(9)

1.5.3 Anti PD-1/PD-L1 pathway immunotherapy in the clinic

FDA has approved 2 PD-1 inhibitors that are monoclonal antibodies to treat metastatic melanoma patients and have accelerated the approval to other cancers. Currently one of the PD-1 inhibitors is Nivolumab (OPDIVO, MDX-1106, BMS-936558 and ONO-4538) from Bristol-Myers Squibb and the other PD-1 inhibitor is Pembrolizumab (MK-3475) from Merck[73]. Phase I clinical trial of Nivolumab results were published in 2010 based on 39 advanced cancer patients ranging from metastatic melanoma (N=10), non-small cell lung cancer (N=6), colorectal cancer (N=14), prostate cancer (N=8) and renal cell carcinoma (N=1). Dose-escalation studies had been compared at 0.3, 1, 3, 10mg/kg[74]. Generally, this Phase I clinical trial indicates that Nivolumab was well tolerated in advanced cancer patients, despite serious adverse effect being observed in 1 advanced melanoma patient who received 5 doses Nivolumab at 1mg/kg developing inflammatory colitis[74]. Another Phase I clinical trial of Nivolumab was performed in 2012 in a large advanced cancer cohort (N=296). Overall, the grade 3 or grade 4 adverse effects were observed in 14% of patients. Cumulative response rate is 28% (26/96 patients) in advanced melanoma cohort, 27% (9/33 patients) renal cell carcinoma cohort, 18% (14/76 patients) in non-small cell lung cancer cohort[75]. In addition, Nivolumab received ORR of 87% in treating refractory Hodgkin's lymphoma[54]. Currently Nivolumab has been approved for the treatment of advanced melanoma, squamous non-small cell lung cancer and more recently Nivolumab has been approved by FDA to treat metastatic urothelial carcinoma in February 2017[76].

Another PD-1 inhibitor Pembrolizumab has achieved similar benefits for patients in treating advanced melanoma when compared with Nivolumab[77]. Notably, results from a large Phase III clinical on advanced melanoma revealed that Pembrolizumab demonstrated prolonged progression-free survival and overall survival with less high-grade adverse effects in treating advanced melanoma when comparing with those who treated with ipilimumab, CTLA-4 inhibitor[78]. Pembrolizumab has also extended its application in other solid tumors including non-small cell lung cancer, gastric cancer, colorectal cancer and triple negative breast cancer[78-81]. Pembrolizumab achieved 19.4% ORR in treating advanced non-small cell lung cancer, over 20% ORR in treating advanced urothelial cancers and 22% ORR in treating advanced gastric cancer, 18.5% ORR in treating advanced triple negative breast cancer with manageable adverse effects[52, 53, 55, 80, 82].

In the meantime, PD-L1 mAbs have also been actively developed and have demonstrated promising clinical benefits in multiple cancers including advanced melanoma, non-small cell lung cancer, and advanced urothelial cancer[83]. PD-L1 mAb could block the interaction not only between PD-L1 and PD-1 but also between PD-L1 and other B7-1[84]. Hence, it may have great potential in treating multiple solid cancers and achieve increased overall response rates. A Phase I study of PD-L1 blockade (MPDL3280A) in treating advanced urothelial cancer achieved remarkable control of tumor growth with 43% ORR in PD-L1+ individuals and 11% ORR in PD-L1- individuals respectively[84, 85].

1.6 CTLA-4 inhibitory pathway

1.6.1 Discovery of CTLA-4 and its function

In the pursuit of identifying molecules on the surface of cytotoxic CD8 T cells that may interact with the molecules on antigen presenting cells, in 1987, the CTLA-4 gene was first isolated from cDNA library of mouse activated CD8 T cell and named cytotoxic T lymphocyte antigen-4 (CTLA-4)[86]. Initially, genetic prediction indicated that CTLA-4 might involve in the costimulation of T cells, as its gene was located in the same chromosome as that of CD28 and CTLA-4 shares high degrees of similarity in sequence with CD28[87]. Further studies revealed that soluble CTLA-4 has 20-fold higher avidity to bind with B7 (a group of membrane proteins expressed on activated antigen presenting cells that bind with CD28 or CTLA-4 on T cells) than CD28, which indicated CTLA-4 might be the additional binding partner of B7 apart from CD28[88].

Furthermore, the focus was around the function of CTLA-4 in T cells proliferation. Contradictory interpretations of CTLA-4's function were arisen. It was evident that adding soluble CTLA-4 into the co-culture between CD8 T cells and B7-expressing APCs resulted in the inhibition of T cells proliferation. On the one hand, some researchers thought that soluble CTLA-4's binding to B7 interfered with the interaction between CTLA-4 on the surface of T cells and B7 on APCs[89]. Hence, those researchers concluded that CTLA-4 might serve as a stimulatory receptor binding with B7 and subsequently inducing T cells proliferation in coordinate with CD28[89, 90]. On the other hand, other researchers interpreted CTLA-4 as an inhibitory receptor that may elicit negative signaling in T cells proliferation. The blocking antibodies of CTLA-4 may diminish its inhibition signaling[91]. This interpretation was later

confirmed by knockout mice and from then on CTLA-4 was known as an inhibitory receptor that could negatively regulate T-cells proliferation and function[92]. Furthermore, it has been found that CTLA-4 could bind to CD80 (B7.1) and CD86 (B7.2) with higher affinity than CD28[93-95].

1.6.2 Anti-CTLA-4 in the clinic

mAbs blocking CTLA-4 has shown the enhancement of T cells anti-tumor responses [96]. Currently 2 CTLA-4 inhibitors, ipilimumab and tremelimumab have been undergoing clinical testing from 2000[97]. Since positive benefits from CTLA-4 were achieved in multiple clinical trials in treating melanoma[98-101]. In 2011, the mAb blocking CTLA-4, ipilimumab, was approved by FDA for the treatment of advanced melanoma, which officially opened the usage of inhibitory pathway blockade in cancer treatments[102, 103]. Of note, in the Phase III clinical trial, ipilimumab significantly increased the long term survival of advanced melanoma individuals than gp100 peptide vaccine. Advanced melanoma has poor prognosis where the 1 year survival rate is only 25%[104]. Treating with ipilimumab in advanced melanoma individuals prolonged their 1 year overall survival to 45.6% and 2 year survival to 23.5% respectively[47]. However, in comparison with PD-1 inhibitors, CTLA-4 antagonists brought severe immune-related adverse effects. It has been reported that ipilimumab responses correlated with its toxicity[105]. In the retrospective review of 14 clinical trials involving 1498 patients, it demonstrated that nearly all the patients have suffered adverse effects with an incidence of 96.6%[104]. In addition, unlike, PD-1 inhibitors in which PD-L1 may predict who would benefit

from PD-1 blockade immunotherapies, currently, no proposed biomarkers have been validated or indicated as the biomarkers to predict who may benefit from CTLA-4 antagonists.

1.7 Tim-3 inhibitory pathway

1.7.1 Expression of Tim-3 and its ligands

T cell immunoglobulin-3 (Tim-3) was first discovered in 2002 on IFN-gamma producing CD4 (Th1) T cells and CD8 cytotoxic T cells[106]. It has been found to express on Tregs, NK cells, monocytes and dendritic cells as well[107] In 2005, one of its ligand, Galectin-9 was identified that could interact with Tim-3 inducing cell death, which indicates that Tim-3 /Galectin-9 pathway may be involved in peripheral tolerance to ensure the termination of effector Th1 cells [108]. In 2007, phosphatidylserine (PtdSer) has been suggested as an additional ligand of Tim-3[109, 110]. However, PtdSer could not only bind to Tim-3 but also could interact with other receptors in Tim families such as Tim-1 and Tim-4[110]. More recently, high mobility group protein B1 (HMGB1) and Ceasam-1 were identified as the ligands of Tim-3 and its binding with Tim-3 could induce negative effector T cells function[111-113]. The interaction between Tim-3 and its currently identified ligands was demonstrated in Figure 1-8.

Figure 1-8: Interaction between Tim-3 and its ligands

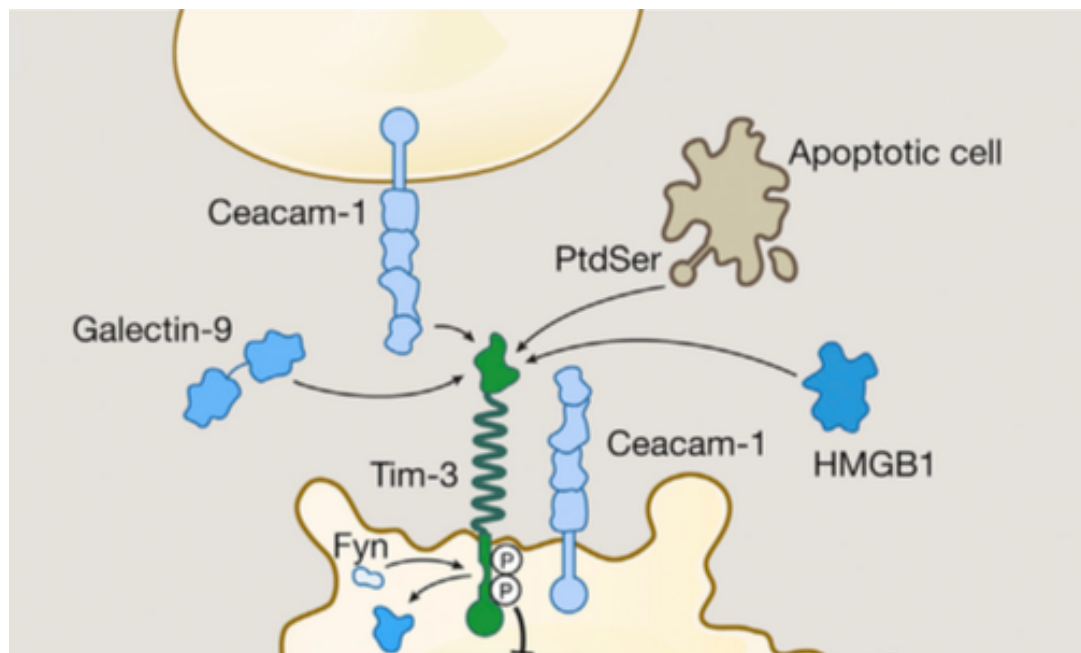


Figure 1-8 was adapted and modified from Anderson et al; *Immunity*, 2016

1.7.2 Tim-3 pathway in diseases

Tim-3 was first found in the establishment of autoimmunity. In animal models, anti-Tim-3 antibodies in mouse were shown to develop experimental autoimmune encephalomyelitis (EAE)[106]. Further experiments then indicated that activation of the Tim-3 pathway contributes to T –cell exhaustion in chronic virus infection and cancer[114, 115]. In HIV or HCV infection, the presence of Tim-3 on antigen specific CD8 T cells is associated with dysfunctional phenotype of T cells and the blockade of Tim-3 could resuscitate the anti-virus responses of effector cells[116, 117]. In addition, the expression of Tim-3 on T cells correlates with viral loads in HIV, HCV or HBV chronically infected individuals[114, 116, 118].

Moreover, the co-expression of Tim-3 with PD-1 defines a more exhausted phenotype of virus specific T cells, which suggests the combinatorial blockade of PD-1 and Tim-3 to restore exhausted T cells in multiple chronic virus infection including HBV, HCV and HIV chronic infection[115, 119, 120]. In cancer tissues, the co-expression of Tim-3 and PD-1 also indicates the severe dysfunctional phenotype of TILs that were unable to produce IL-2, IFN-gamma and TNF[121]. As more individuals with advanced cancer were treated with PD-1 inhibitors, some adaptive resistance of PD-1 inhibitors has been observed in a number of patients. 25% of advanced melanoma patients who have shown responses to anti-PD-1 blockade immunotherapy had disease progression after 2 years[122]. Recent studies suggested that in advanced lung cancer patients who developed adaptive resistance to anti-PD-1 blockade, Tim-3 expression was up-regulated on both CD4 and CD8 TILs[123]. Its up-regulation was mainly observed in anti-PD-1-antibody-bound TILs when adaptive resistance of anti-PD-1 was developed[123]. Since Tim-3 expression was unregulated on the anti-PD-1 treatment failure TILs, which may be a more exhausted phenotype of TILs, this result is in line with the functional studies of antigen specific T cells in chronic virus infection where co-expression of PD-1 and Tim-3 indicated severe T-cell exhaustion phenotype. These findings suggested the combinatorial blockades of PD-1 and Tim-3 inhibitory pathways and implies that in anti-PD-1 adaptive resistance individuals, alternative blockade such as Tim-3 pathway may sustain the anti-cancer responses and limit disease progression. In all, Tim-3 pathway may have great potentials as an additional target to block and treat advanced cancers.

1.8 KLRG-1 inhibitory pathway

1.8.1 Expression of KLRG-1 and its ligands

In both mice and humans, the killer cell lectin like receptor G1 (KLRG-1), was identified to express on NK cells[124]. Further studies also revealed that it expresses on antigen experiencing T cells and a subset of gamma/delta T cells[125, 126]. Initially functional studies indicated that KLRG-1 is associated with T cells differentiation[127, 128]. It has low expression on naïve T cells while its expression is upregulated on central memory and effector memory phenotypes. Hence, it was applied as a T-cell differentiation marker to distinguish different differentiation stages along with CD45RA, CCR7 and CD27[129]. It is of note that KLRG-1 has high expression on terminally differentiated T cells along with the presence of CD45RA[130]. Furthermore, KLRG-1 was also an inhibitory receptor on both NK cells and T cells negatively regulating effector cells when interacting with its ligands[131]. 3 ligands were identified that could bind with KLRG-1 in different affinities inducing inhibitory function of effector cells, and these are e-cadherin, n-cadherin and v-cadherin[132, 133]. In vitro experiments suggested that the blockade of KLRG-1/e-cadherin pathway restores its inhibitory effect on CD8 T cells with enhanced proliferative activity. In addition, KLRG-1 expression is also correlated with aging. It has been reported that in young healthy individuals, KLRG-1 expressed on about 40% CD8 T cells and 20% CD4 T cells respectively in the periphery. Its frequency would be increased to 90% of in aged healthy individuals over 65 year-old[134, 135]. Recently, studies also indicated that expression of KLRG-1 on NK cells has a key role in cancer immunosurveillance to prevent the metastasis[136].

1.8.2 KLRG-1 inhibitory pathway and its role in cancer

KLRG-1 has dual functions as a T-cell differentiation marker and a co-inhibitory receptor. Upon cognate antigen recognition through MHC-I/TCR complex, CD8 T cells differentiated into effector phenotype with enhanced proliferative activity. Naïve T cells were activated through CD28, which further initiates Akt signalling pathway resulting to the proliferation of T cells[137, 138]. In the transition from naïve T cells to effector T cells, CD8 cells lose the expression of CD28 while upregulating the expression of the co-inhibitory receptor, KLRG-1, which then inhibits the Akt phosphorylation through SHIP-1 and SHP-2[139]. The blockade of KLRG-1 in differentiated CD8 T cells reverses the inhibitory function KLRG-1 exerted and restores the phosphorylation of Akt signalling resulting to the enhancement of proliferation activity of dysfunctional CD8 T cells[135, 140]. The mechanism is illustrated in Figure 1-9.

Figure 1-9: KLRG-1 inhibits CD8 T cells function through Akt signalling

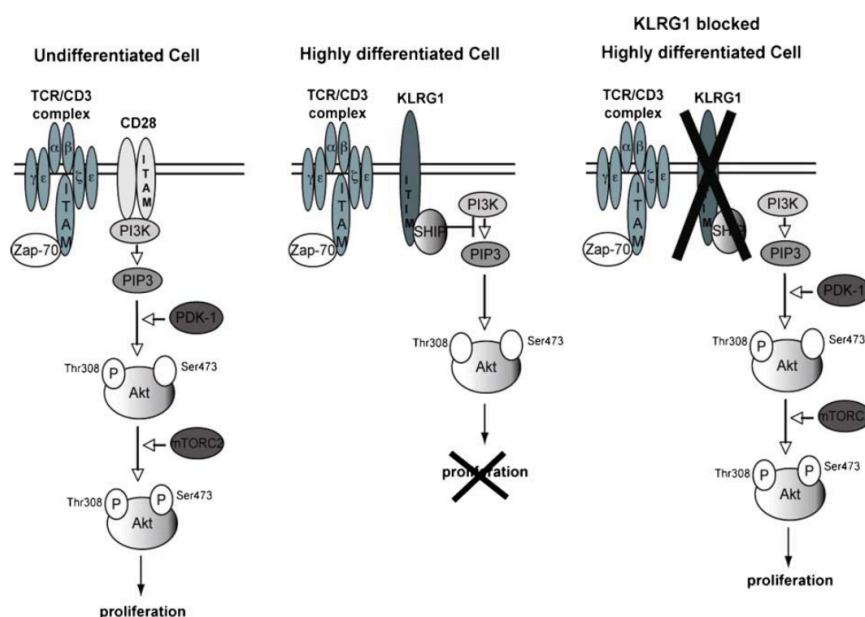


Figure 1-9 was adapted from Henson et al; Age, 2009

In chronic HCV, HBV or HIV infection, multiple inhibitory receptors were upregulated on virus specific T cells with impaired function, which are proposed as the markers of T-cell exhaustion. KLRG-1 has been found to have an upregulated expression on virus specific CD8 T cells in chronic HCV and HBV infection[29, 141]. In advanced melanoma individual, KLRG-1 is upregulated on tumor specific T cells in tumor infiltrated lymph nodes and co-expresses with PD-1, 2B4 and Tim-3[142]. In addition, upregulation of KLRG-1 has also been seen in bulk CD4 and CD8 T cells in colon cancer and ovarian cancer individuals in the periphery[143]. In the tumor microenvironment, KLRG-1 is upregulated on TILs and KLRG-1+CD8 TILs co-expresses CD73 and CD39, the immunosuppressive markers. This indicates KLRG-1+ CD8 TILs may contribute to the immunosuppressiveness in tumor microenvironment[143]. It also implies that in the combinatorial blockade of multiple inhibitory pathways, KLRG-1 may be a candidate pathway, but concerns should be noticed as it regulates NK cells function as well.

1.9 2B4 pathway

1.9.1 Expression of 2B4 and its ligand

CD244 (2B4) is molecules belong to CD2 family and expressed on NK cells, T cells, basophils and monocytes[144]. Currently, the full function of 2B4 pathway in humans is yet to be elucidated as it has dual functions, acting as both inhibitory receptor and stimulatory receptor. It has a binding ligand, CD48 that expresses on nearly all hematopoietic cells[145].

1.9.Dual roles of 2B4

Initially in murine studies, 2B4 was proposed to be a stimulatory receptor as the presence of anti-2B4 resulted in the increased cytotoxic function and IFN- γ production in NK cells[146]. However, other studies also demonstrated that the 2B4 may also have inhibitory function as the absence of 2B4 or CD48 on target cells enhances the cytotoxic function of NK cells[147, 148]. Hence, 2B4 has a dual functions acting as both activating receptor and inhibitory receptor. In human studies, it was found that 2B4 had dual functions as well. It was shown that 2B4 could act as the ligand rather than receptor on target cells, which interact with CD48 expressing NK cells activating human NK cells cytotoxic functions[149]. In HIV infection, it was found that the 2B4+CD8 virus specific T cells had impaired cytokine production and may play an inhibitory role in the insufficient control of chronic virus infection[150]. However, more recently in the comparison of HIV elite controllers and chronic progressors, it was found that the co-expression of CD160 and 2B4 delineates a subset of CD8 T cells with high capacity of perforin production in elite controllers but this subset was absent in chronic progressors[151]. In addition, in chronic HCV and HBV infection, it was found that 2B4 co-expressed with PD-1 on virus specific CD8 T cells demonstrating dysfunctional cytotoxic functions[29, 141].

In all, 2B4 pathway is complex as it could be act as both receptor and ligand inducing either stimulatory signal or inhibitory signal. The dual functions of 2B4 on T cells and NK cells may play an important role in virus control and cancer.

1.10 TIGIT inhibitory pathway

1.10.1 Expression of TIGIT and its ligands

Initially the discovery of T cell Ig and ITIM domain (TIGIT) was predicted from bioinformatics algorithms[107]. TIGIT was a newly identified inhibitory receptor expressing on NK cells, Tregs, effector and memory T cells[152-154]. Currently 2 ligands that interact with TIGIT were identified, CD112 and CD155. TIGIT has higher affinity in binding with CD155 than that in CD112[107, 155]. Most of the studies were conducted on the interaction between TIGIT and CD155 while, little was known between TIGIT and CD112. The inhibitory function induced through TIGIT was quite similar to that of CTLA-4. The binding between CD226 and CD155 could deliver stimulatory signal of T cells and NK cells[156]. However, TIGIT competitively binds with CD155 with much higher affinity than CD226[157]. The binding between TIGIT and CD155 delivers inhibitory signal to T cells and NK cells[157]. This mechanism is illustrated in Figure 1-10. TIGIT interferes the binding between CD226 and CD155 in a dose dependent manner[152, 153]. As the ligands of TIGIT, it was found that CD112 and CD155 are widely expressed on APCs and cancer cells in tumor microenvironment[158, 159]. In addition, CD96 also enables to bind with CD155 inducing inhibitory signal[157].

Figure 1-10: Interaction between TIGIT and its ligands

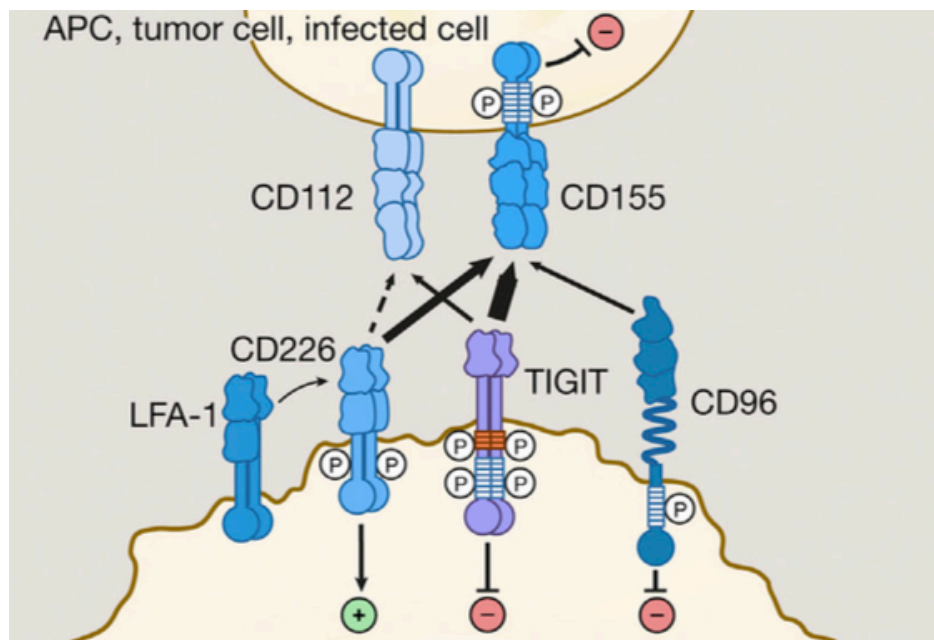


Figure 1-10 was adapted from Anderson et al; Immunity, 2016

1.10.2 TIGIT inhibitory pathway in chronic virus infection and cancer

TIGIT is highly expressed on TILs in tumors of both murine and human samples[160]. More importantly, one animal study revealed that TIGIT had higher expression on TILs in tumors but relatively low expression on T cells in the peripheral lymphoid organs in tumor bearing mice, which indicates its upregulation may result from the modulation of tumor microenvironment and thus had great potentials to target[160]. In the same study, it also demonstrated that TIGIT was co-expressed with PD-1, Tim-3 and Lag-3 on CD8+ TILs, and marks the dysfunctional phenotype of TILs[107]. In animal experiments, it was shown that TIGIT deficient mice demonstrated delayed tumor growth[160]. In human studies, it was shown that 1: CD8 TILs had lower expression of CD226, the costimulatory receptor, while higher expression of TIGIT; 2: the co-blockade

of TIGIT and PD-1 additively restored the dysfunctional tumor specific CD8 TILs in advanced melanoma individuals[161].

1.11 CD160 and BTLA inhibitory pathways

1.11.1 Discovery of CD160 and BTLA and their ligand

CD160 was first discovered on NK cells by monoclonal antibody BY55[162]. The expression of CD160 has been found on CD8 T cells, cutaneous T cells, CD4 T cells and activated endothelial cells.[163-165]. It was proposed as an activating receptor on CD56dim NK cells[166]. Soluble form of CD160 was detected releasing from activated NK cells in human peripheral blood. This soluble form of CD160 could inhibit NK cells' cytotoxicity[167]. B-lymphocyte and T-lymphocyte attenuator (BTLA) was identified by the screening of gene expression of CTLs and T cells undergoing apoptosis[168]. It was shown that BTLA constitutively expressed on CD4 and CD8 T cells and its expression decreases upon the activation of T cells[169]. Herpes virus entry mediator (HVEM) was identified as the ligand for both BTLA and CD160[170]. HVEM is widely expressed on a variety of cells including lymphocytes, endothelial cells and APCs[171]. Its interaction with CD160 and BTLA could induce inhibitory signal from which it diminished T cells activation[170]. The inhibition through HVEM and its inhibitory receptors resulted in dysfunctional antitumor responses[172-174]. HVEM could also bind with LIGHT (homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T

lymphocytes) to elicit stimulatory signal. Hence, HVEM has bidirectional functions in modulating T cells activation[175]. The dual functions of HVEM pathway are illustrated on Figure 1-11.

Figure 1-11: Dual function of HVEM pathway

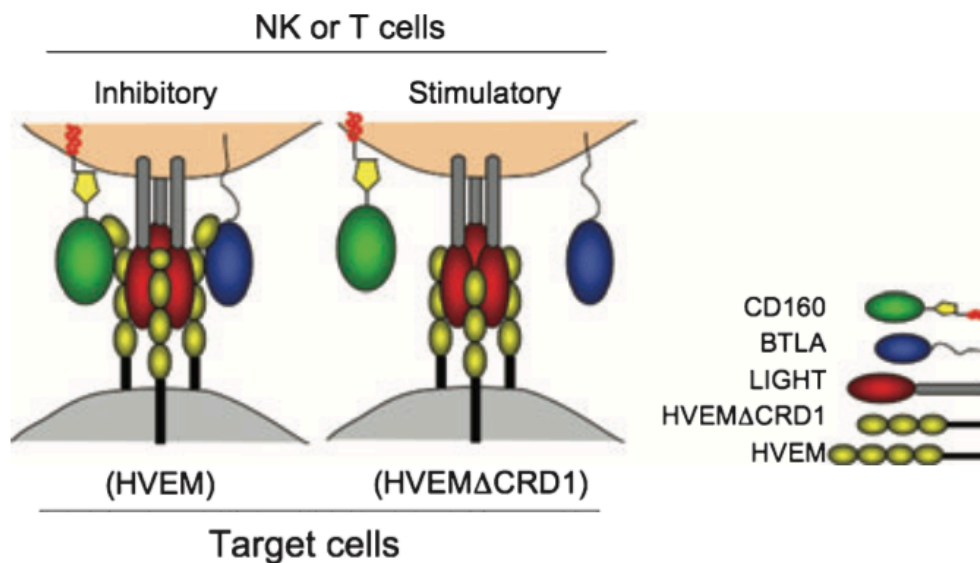


Figure 1-11 was adapted from Cai et al, Immunological reviews 2009

1.11.2 CD160 and BTLA pathways in cancer

The inhibitory signal through the interaction between HVEM and BLTA/CD160 has been investigated on several studies. It was shown that BLTA was overexpressed in tumors and BTLA-deficient mouse develops severe autoimmunity[176]. This result suggests that tumor microenvironment may hijack this pathway to evade anti-cancer immune responses. The blockade could be achieved on targeting Cysteine-rich domain (CRD1), shown on Figure 1-11, the BTLA/CD160 binding domain of HVEM, as the blockade of this domain would not affect the interaction between LIGHT and HVEM[175]. In human studies, it was found that BTLA was gradually downregulated from naïve

phenotype to memory phenotype through the differentiation of influenza specific CD8 T cells[177]. However, BTLA persistently maintained high expression on tumor specific CD8 T cells[178]. This indicates the blockade of HVEM and BTLA/CD160 pathways may restore anti-cancer T cell responses, although more functional studies are required to further describe its role in tumor immunity.

1.12 Molecular pathways of inhibitory receptors in T cells

Overall, the precise downstream molecular pathways of inhibitory receptors in effector immune cells are poorly deciphered[179]. The downstream signaling of CTLA-4 and PD-1 are the best-characterized molecular pathways of inhibitory receptors. Upon binding with CD80 or CD86, the cytoplasmic domain of CTLA-4, YVKM motif recruits 2 phosphatases SHP-2 and PP2A as shown in Figure 1-12[180, 181]. The SHP-2 recruitment results in the dephosphorylation of CD3 ζ chain and thus attenuating TCR activation signaling. In addition, the PP2A recruitment by CTLA-4 results in dephosphorylation of AKT, by which it further dampens T-cell activation[182]. PD-1 has a cytoplasmic Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM), as well as an Immunoreceptor Tyrosine-based Switch Motif (ITSM). PD-1 ligation by PD-L1 or PD-L2 recruits the phosphatases SHP-1 and SHP-2 to the ITIM domain resulting the attenuation of TCR signaling[183, 184].

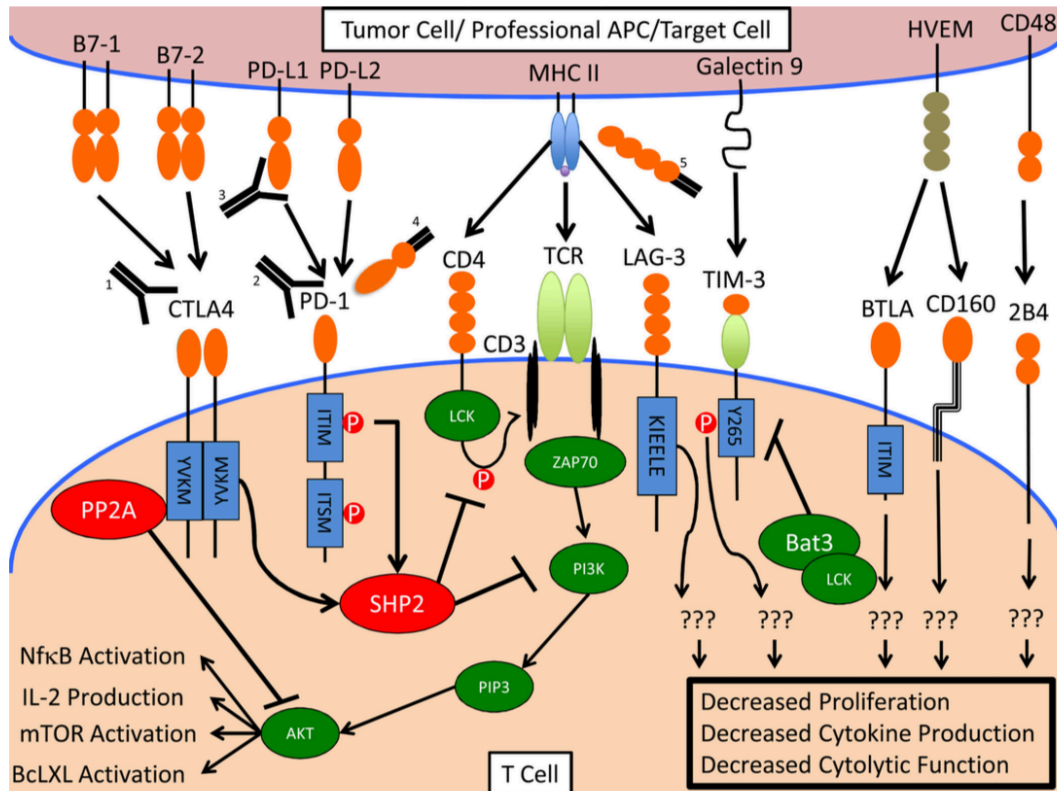


Figure 1-12 was adapted from Cristopher et, al; Clin Cancer Res. 2013

Tim-3 downstream signaling is dependent on the phosphorylation of Y265 (the intracellular domain of Tim-3)[185]. Tim-3 has an intracellular binding protein-HLA-B-associated transcript 3, (Bat3), which bound to Tim-3 at resting state preventing the activation of Tim-3 signaling[186]. However, recent discoveries indicate that upon the binding with Galectin-9, Y265 is phosphorylated and Bat3 dissociates from Tim-3[187]. Thereafter, Tim-3 signaling down-regulates T-cell proliferation and cytokine production shown on Figure 1-12, 1-13.

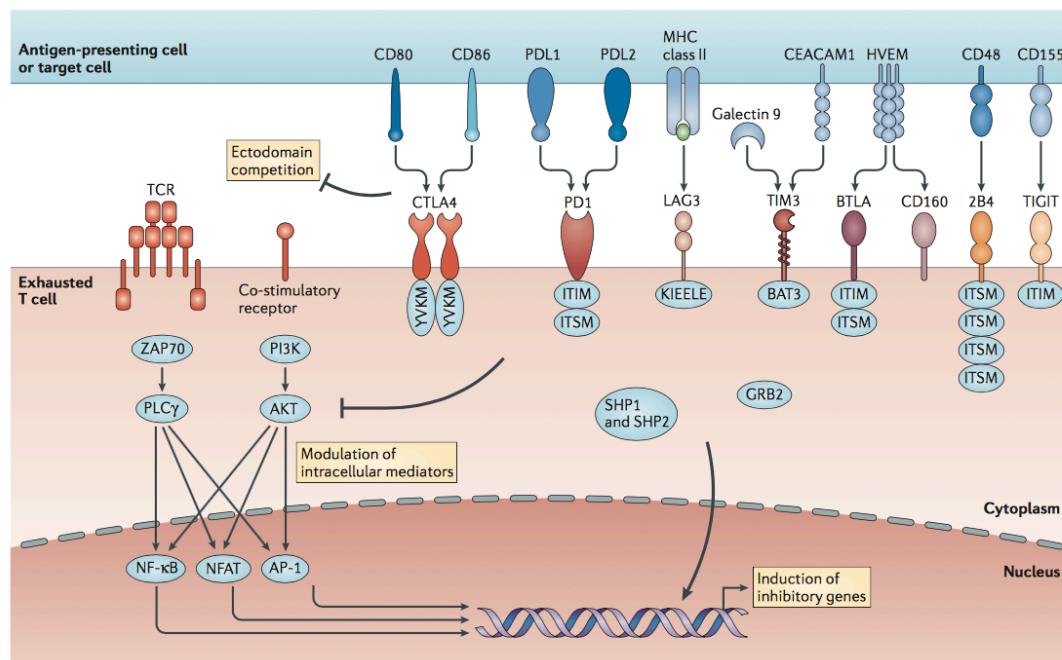


Figure 1-13 was adapted from E. John Wherry, Nat Review Immunology 2015

Both BTLA and CD160 bind with HVEM eliciting inhibition signaling. BTLA contains both ITIM and ITSM domains while the intracellular domains of CD160 are not fully characterized yet[179]. The intracellular signaling of 2B4 upon binding with CD48 is poorly understood as well.

In summary, multiple known inhibitory receptors on T cells have ITSMs and / or ITIMs in their intracellular domains that may involve in the inhibitory signalling, although some receptors have specific domains, such as YVKM for CTLA-4. The molecular pathways of inhibitory receptor signalling can be subdivided into 3 categories shown on Figure 1-13: 1: ectodomain competition which elicits the attenuation of stimulatory signalling; 2: modulation of intracellular mediators; 3:

induction of inhibitory genes through nuclear factor- κ B (NF- κ B), nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1) in the nucleus[187].

Hypotheses to test and aims of this thesis:

Chapter 3:

Hypothesis 1: Tumor microenvironments specifically up-regulate multiple inhibitory receptors on TILs when comparing with its counterparts in the blood

Hypothesis 2: The expression of multiple inhibitory receptors on TILs correlate with its expression on PBMCs in HBV HCC and breast cancer individuals

Hypothesis 3: Different cancers have differential mono-blockade immunotherapy regimens based on its expression on TILs

Hypothesis 4: Different cancers have differential combinatorial-blockade immunotherapy regimens based on the co-expression patterns of multiple inhibitory receptors on TILs

Aims:

1: Identify the correlation between the expression of inhibitory receptors on T cells from peripheral blood and tumor tissues of different cancer individuals;

2: Dissect the expression of inhibitory receptors on T cells from different tumor microenvironment;

3: Characterize the co-expression pattern of inhibitory receptors on T cells in different cancers.

Chapter 4:

Hypothesis 1: The expression of inhibitory receptors on TILs positively correlate with the ligands expression in tumor microenvironment

Aims:

- 1:** Characterize the expression pattern of PD-L1, Galectin-9 and e-cadherin from 39 paired breast cancer individuals;
- 2:** Quantify the expression of PD-L1, Galectin-9 and e-cadherin from 39 paired breast cancer individuals in disease stage based manner;
- 3:** Identify the correlation between the expression of PD-L1, Galectin-9 and e-cadherin and its inhibitory receptors on CD4 and CD8 T cells from 39 paired breast cancer individuals.

Chapter 5:

Hypothesis 1: There is distinctive expression of inhibitory receptors on circulating lymphocytic cells within different stages of chronic HBV disease.

Aims:

- 1:** Identify the distinctive mono-or combinatorial expression of inhibitory receptors on circulating T cells from individuals in different HBV associated disease stages
- 2:** Characterize the expression of multiple inhibitory receptors on different subsets simultaneously

2 Chapter 2: Material and Methods

2.1 Study cohort and healthy donors

Ethical approvals were obtained prior to the study from Scientific and Ethical Committee of Beijing You'an Hospital, Capital Medical University in Beijing, China and Clinical Research Ethical Committee of Xinjiang Cancer Hospital, Xinjiang Medical University in Urumqi, China. Details of the ethical approvals attached in the Appendix 1 respectively. All the patients were fully consented before prior the collection of clinical samples.

2.1.1 Beijing HBV associated HCC cohort, China

From 2012, 66 HBV chronically infected individuals from Beijing You'an Hospital were recruited in the Beijing HBV associated HCC cohort, ranging from HBV chronically infected individuals, HBV chronically infected liver cirrhosis individuals and HBV chronically infected HCC individuals. Either patient's blood or tissue samples were obtained for this study. Ethics approval was attached in the Appendix 2.

2.1.2 Xinjiang cancer cohort, China

From 2014, 95 individuals were recruited in Xinjiang Cancer Hospital for this study in which it consists liver cancer patients, hepatic hemangioma patients, esophageal cancer patients, colorectal cancer patients, gastric cancer patients, thyroid cancer patients, breast cancer patients, breast fibroadenoma patients lung cancer patients and kidney cancer patients. Either patient's blood or tissue samples were obtained for this study. Ethics approval was attached in the Appendix 3.

2.1.3 Oxford healthy donors cohort, UK

From 2012, 25 healthy individuals were recruited as the healthy controls to this study. They are either students or staffs at the Weatherall Institute of Molecular Medicine. Their gender ratio and ethnicity are matched to the abovementioned research cohorts.

2.1.4 Inclusive and exclusive criteria of surgical samples from multiple cancer individuals

Sample types:

1. Fresh venous blood;
2. Cryopreserved PBMCs;
3. Paraffin embedded tumor tissues/adjacent non-cancerous tissues;
4. Frozen tumor tissues/adjacent non-cancerous tissues;

Inclusive criteria:

1. Primary hepatocellular carcinoma patients who underwent partial hepatectomy or liver biopsy;
2. Hepatic hemangioma/benign lesion resection;
3. Primary renal carcinoma patients who underwent partial nephrectomy or total nephrectomy;
4. Primary gastric cancer patients who underwent partial gastrectomy, total gastrectomy, sleeve gastrectomy or oesophagogastrrectomy;
5. Primary breast cancer patients who underwent standard mastectomy, subcutaneous mastectomy, radical mastectomy or skin-sparing mastectomy;

6. Primary colorectal cancer patients who underwent partial colectomy, total colectomy, hemicolectomy or proctocolectomy;
7. Primary thyroid cancer or benign thyroid lesion disease patients who underwent partial thyroidectomy, subtotal thyroidectomy, total thyroidectomy, hemithyroidectomy;
8. Primary oesophageal cancer patients who underwent partial oesophagectomy or total oesophagectomy;
9. Primary melanoma patients who underwent skin surgery;
10. Primary cervical cancer patients who underwent trachelectomy;
11. Primary lung cancer patients who underwent pneumonectomy, lobectomy, wedge resection or segment resection;

Exclusive criteria:

1. HCV+ patients in any type of cancers or benign lesion removal surgeries;
2. HIV+ patients in any type of cancers or benign lesion removal surgeries;
3. Any metastatic cancer patients;
4. Any type of recurrent cancer patients;
5. Post chemotherapy or radiotherapy of any primary cancer patients;
6. Primary cancer patients with autoimmune diseases;
7. Any type of cancer patients who have received transarterial chemoembolization procedures (TACE) before inclusion;
8. Any patients who have received organ transplantation surgeries;

2.2 Peripheral blood mononuclear cell separation

Fresh Blood was collected in the EDTA spray-dried tube by the vacutainer kit (BD catalog No. 367861). Peripheral blood mononuclear cells (PBMCs) were separated within 6 hours after collection. Blood in the tube was diluted by room temperature RPMI 1640 (Sigma-Aldrich, Dorset, UK) at 1:1 ratio. Then it was transferred to BD 50ml falcon tube (Corning, Inc. catalog No. 352070) containing 15ml lymphoprep solution. Further it was centrifuged at 2500RPM 25mins with brakes. PBMCs were harvested from the gradient interface and washed twice in R10 (RPMI 1640 supplemented with 10% fetal calf serum (FCS; Sigma), 100IU of penicillin-L, 100mg of streptomycin-L and 2mM L-glutamine). Cell counting was conducted with trypan blue (Invitrogen, California, USA) under light microscope. Half of the cells were stained for *ex vivo* phenotypic analysis and half was cryo-preserved in 10% DMSO (dimethylsulphoxide)/FCS at 5 million cells per tube aliquot in liquid nitrogen.

2.3 Optimization of intrahepatic lymphocytes isolation

Previous studies have indicated that healthy liver enriches numerous intrahepatic lymphocytes acting normal immune function inside the liver. Sole mechanical dissociation method has very limited yield of intrahepatic lymphocytes. Based on the results of 7 liver samples from liver transplants in October to December 2012, on average, the mean yield is about 0.23×10^6 cells from 1g liver tissues (data not shown). Particularly in those who have severe liver cirrhosis, less intrahepatic cells could be isolated. However, we need large quantity of viable and purified IHLs after designing new flow cytometry panels in 2013. We therefore used Octo-Gentle MACs dissociator (Miltenyi Biotech,

product No: 130-095-937) and MACs tube rotator (Miltenyi Biotech, product No: 130-090-753) together with Collagenase to dissolve liver tissues and tumor tissues in order to increase the yield of IHLs. Dissociator-based enzymatic treatment improves the yield of intrahepatic lymphocytes (IHL). In enzymatic treatment, Collagenase A, Collagenase D and Liberase at the recommended working concentration (1mg/ml, 1mg/ml and 0.35mg/ml respectively) can better isolate IHLs than sole mechanical dissociation with a mean yield of 0.48×10^6 cell in 1g liver tissues from 4 samples in May to June 2013 (data not shown). However, hardly could we obtain satisfied yield ($<0.4 \times 10^5$ tumor-infiltrating lymphocytes/g tumor sample) from both methods in tumor infiltrating lymphocytes. We then increased the working concentration of Collagenase D from 1mg/ml to 2mg/ml to isolate the tumor infiltrating lymphocytes but still we did not obtain great quantity of tumor infiltrating lymphocytes. Thereafter, from September to December 2013, a human tumor dissociation kit (Miltenyi Biotech, product No: 130-095-929) was tested for the isolation of tumor infiltrating lymphocytes. The mean yield of IHLs from 2 patients is 12.13×10^6 cells from 1g tumor center tissues (Data presented on Table 2.1 and Table 2.2). We demonstrate the differences in flow cytometry between mechanical and dissociator-based enzymatic treatment (shown on Figure 2.1).

Table 2.1:

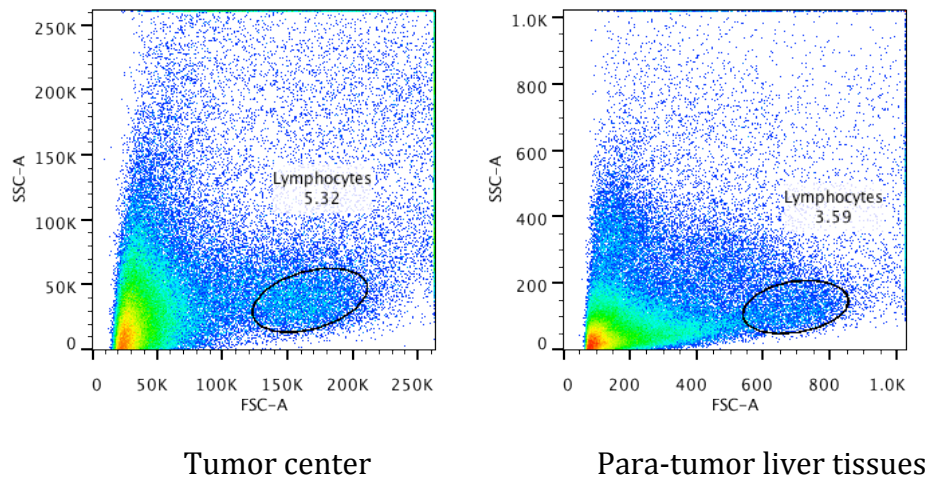
Patient A	Tumor center samples	Tumor edge samples	Para-tumor liver samples	Remote liver samples
Weight of samples	1.54g	3.3g	4.84g	4.8g
Yield of lymphocytes	20×10^6 cells	25×10^6 cells	4×10^6 cells	2×10^6 cells
Yield of lymphocytes/gram	12.99×10^6 cells/g	7.5×10^6 cells/g	0.83×10^6 cells/g	0.42×10^6 cells/g

Table 2.2:

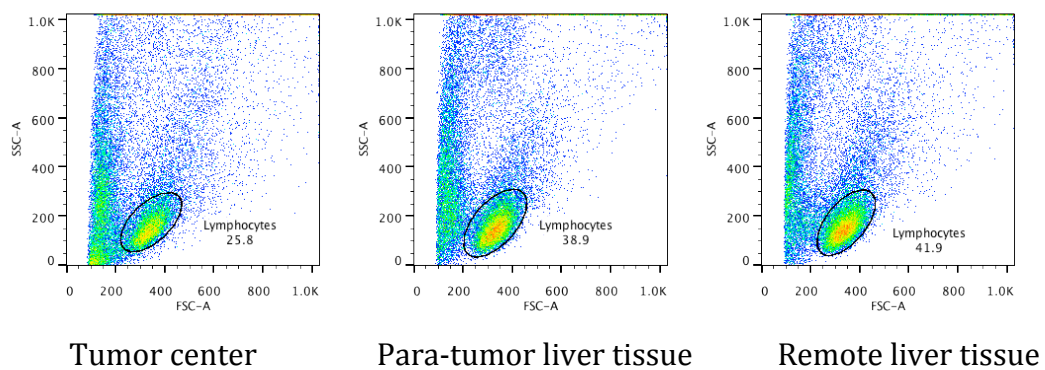
Patient B	Tumor center samples	Tumor edge samples	Para-tumor liver samples
Weight of samples	13.32g	6.17g	14.81g
Yield of lymphocytes	150*10 ⁶ cells	51*10 ⁶ cells	14*10 ⁶ cells
Yield of lymphocytes/gram	11.27*10 ⁶ cells/g	8.3*10 ⁶ cells/g	0.97*10 ⁶ cells/g

Figure 2.1: Comparison of Mechanical treatment & Enzymatic treatment

In mechanical treatment both tumor tissues and liver tissues were dissolved without Collagenase



In enzymatic treatment, tumor tissues were dissolved by the human tumor dissociation kit and liver tissues were dissolved by Collagenase D



However, due to limited samples, we didn't measure cell viability or surface molecules' expression after using 3 types of Collagenase, but this is an opportunity for further optimization. Furthermore, previous study has also systematically evaluated the effect of collagenase D on cell's viability. According to those results, within 30mins the cell's viability and surface markers' expressions would not change significantly compared to mechanical dissociation[188]

2.4 Mechanical and enzymatic dissociation of intrahepatic lymphocytes

2.4.1. Isolation of intrahepatic lymphocytes

In order to investigate IHLs and its function in HBV infection as well as its relationship with carcinogenesis, a stable and reliable technique for the isolation of intrahepatic lymphocytes is necessary. Most of the experimental protocols for the isolation of intrahepatic lymphocytes are based on animal models, which include both mechanical and enzymatic methods[189]. However, there is still a gap in human intrahepatic lymphocytes isolation experience, which is not quite optimized due to the frequencies of obtaining such precious samples and the use of non-standardized protocols modified from the isolation of murine hepatic lymphocytes. Therefore, at the first stage, we estimated and optimized the isolation methods of human liver tissues while conducting phenotypic analyses. From June to July 2013, 4 samples were obtained from chronic HBV related HCC patients who underwent liver transplants in the Beijing You An hospital. We got

standardized consent forms signed by patients (template of consent form shown in the Appendix). The full consent form is attached in the appendix.

2.4.1.1 Mechanical Isolation of intrahepatic lymphocytes

From 2012 to 2013, samples from 44 HCC patients who either underwent partial hepatectomies or liver transplants were collected and mechanically treated. The samples (1 cm³ each) were collected from both the tumor center and para-tumor tissues (3 cm away from tumor edge).

Procedures of mechanical dissociation method are listed below.

- (1) Either tumor or liver tissues were weighed with an electronic balance
- (2) Finely chopped to 1-3mm pieces, and suspended in 20ml RPMI in 50ml tubes in the shaker at room temperature for 30mins
- (3) Washing twice by RPMI, cells were centrifuged at 1600rpm for 5mins
- (4) Cells were re-suspended in 8ml RPMI and transferred to 5ml lympho-prep containing 15ml tubes, which were centrifuged for 30mins at 2500rpm without braking
- (5) The lymphocyte-layer was transferred using a pastuer, into a 15ml tube and washed by RPMI twice
- (6) Finally, the cells were counted by cell counter machine to calculate the average yield
- (7) FACS staining

2.4.1.2 Enzymatic Isolation of intrahepatic lymphocytes

At the second stage experiments during June to July 2013, four samples were obtained from chronic HBV related HCC patients who underwent liver transplants in the Beijing You An hospital. Enzymatic dissociation method was first attempted. Para-tumor tissues were collected and used to optimize the yield of IHLs using collagenase A, collagenase D and liberase. Keeping the same distance from the tumor edge, we incised 3 same size para-tumor tissues around the tumor of each liver. Dissociation procedures are listed.

- (1) Every sample was weighed with an electronic balance
- (2) Liver tissues were finely chopped to 1-3mm pieces, and transferred to C tubes (Miltenyi Biotech, product No: 130-093-237) with different enzyme containing solutions (diluted in RPMI at certain concentration) The weight of tumor is up to 4g per C tube.
- (3) Run different programmes according to the tissue types in Gentle MACs dissociator.
- (4) Incubate the tissues in Gentle MACs rotator in 37°C, 5% CO₂ for 30mins.
- (5) Then it was followed by 2 steps 10ml RPMI washing, in which 70um and 40um cell strainers were used to purify intrahepatic immunological cells.
- (6) Finally, to further isolate IHLs, cells were re-suspended in 8ml RPMI and transferred to 5ml lympho-prep containing 15ml tubes, which were centrifuged for 30mins at 2500rpm without braking.
- (7) Thereafter, the lymphocyte-layer was transferred using a pastuer, into a 15ml tube and washed by RPMI twice.

(8) Finally, the cells were counted by cell counter machine to calculate the average yield.

(9) Cells were ready for staining or cellular assays

2.3 Human tumor dissociation and immune cells separation

Surgical tissues were immediately transferred to tumor dissociation solution (miltenyi biotec, catalog No. 130-095-929) containing C tube (miltenyi biotec, catalog No. 130-093-237). Then the tissues were dissected into 1-3mm pieces by sterile surgical scissor (Ethicon, USA). C tubes were placed on Octo-gentle dissociator (miltenyi biotec, catalog No. 130-095-937). Human tumor program-1 was performed for the dissociation followed by 20mins incubation on the Gentle-mix rotator (miltenyi biotec, catalog No. 130-090-753) at 37°C, 5% CO₂ incubator. Then 70nm cell strainer (Sigma-ALdrich, Dorset, UK) was applied to purify the intra-tumor or intra-tissue lymphocytes. Further, cells were washed twice in R10 and counted by trypan blue staining. Detailed protocol is attached in the Appendix3.

(1) Transfer the tumor tissues taken from the surgeries to 5 ml room temperature RPMI or tissue storage solution (Miltenyi Biotech 130-100-008) in 50ml BD Falcon tubes

(2) Transport the tissues from O.R to lab as soon as possible (within 30mins), then weigh the tissues respectively and fill in tissue dissociation form in detail

- (3) Make liver dissociation solution and tumor dissociation solution respectively. Liver dissociation solution: dissolve Collagenase D (Roche 11088882001) to 1mg/ ml in 37 degree RPMI, then filter the solution with 0.22um strainer to 50ml BD falcon tubes and storage at 4 degree. It could be preserved up to 4 weeks. Warm up at 37 degrees bath when using.
- (4) Tumor dissociation solution: follow the instruction of Human Tumor Dissociation Kit to aliquot Solution 1, Solution2 and Solution3 and store at -20 degrees (Miltenyi Biotech 130-095-929), (1unit formula is for up to 2mg tumor tissues, if the tissues is more than 2mg, add the unit accordingly)
- (5) Use surgical scissors to cut the tissues to 1-3mm pieces in sterile 5ml culture dish (Thermo Scientific 145389), then use 5-10ml liver dissociation solution or tumor dissociation solution to wash the tissues in culture dish and transfer it to C tubes or M tubes for further dissociation (Miltenyi Biotech C tubes 130-096-334, M tubes 130-096-335)
- (6) For tumor tissues, choose H_Tumor_1 program in gentleMACS Octo Dissociator (Miltenyi Biotech 130-095-937) to process the tumor tissues, then put the tubes into the rotator at 37 degrees for 30mins, then run H_Tumor_2 program in gentleMACS Octo Dissociator followed by 30mins 37 degrees culture on rotator again.
- (7) For liver tissues, choose “Xi Li” program in dissociator (the liver is highly cirrhotic, repeat the program once), then put the tubes into the rotator at

- 37 degrees for 30mins. Transfer the solution from C tubes or M tubes to 50ml BD falcon tubes
- (8) Make Tissue Washing Solution, (0.1mg DNase-1/ml in RPMI Roche10104159001), and top up cells containing 50ml falcon tubes with Tissue Washing Solution and centrifuge it at 1700rpm 6mins.
- (9) Gently pour off the supernatant and top up RPMI, centrifuge it at 1700rpm 6mins again. (The pellets of Intrahepatic lymphocytes or tumor infiltrating cells have a loose nature, make sure it is not lost visually when pouring the supernatant)
- (10) Apply 5-10ml 1X red blood cell lysis buffer (BD 555899) to resuspend the cells for 15mins dark at room temperature, then top up RPMI to centrifuge the cells at 1700rpm 5mins
- (11) Count the cells and put it in the incubator waiting for FACS staining

2.4 Multichromatic flow cytometry panels

2.4.1 Panel design for the analysis of inhibitory receptors expression on T cells and T-cell differentiation

Flow cytometry panels were optimized at Oxford on Fortessa (BD) and Fortessa X20 (BD) from 2012. With the upgrade of filters in violet laser channel at Oxford lab and collaborative hospitals in China (both You'an hospital and Xinjiang Cancer Hospital), panels were changed accordingly. Panel 1 to panel 6 were the performed at Beijing You'an Hospital in China from 2014 to 2016. Panel 7 to Panel 10 were performed at Xinjiang Cancer Hospital in China from 2015 to 2016. Currently Exhaustion panel and Differentiation panel are incorporated together at a 14-color panel in Beijing You'an Hospital allowing us to investigate the combinatorial expression analysis. Details of panels are listed below.

Exhaustion Panels were designed to investigate the inhibitory receptors expression on CD4⁺ T cells and CD8⁺ T cells. Specifically, exhaustion panels with period updates (add or delete several markers) intended to characterize its expression on T cells from PBMCs, IHLs and TILs in different stages of HBV associated liver diseases and multiple cancer individuals. In addition, we have also analyzed the differential T-cell differentiation frequencies in HBV associated liver disease progression and other cancer individuals by T-cell differentiation panels. Further, with the preliminary data we have obtained, we designed a 14-color panel to simultaneously investigate the expression of 6 inhibitory receptors along with 5 T-cell differentiation stages.

Panel 1

<i>Panel acted from August 2014 to July 2015 at Beijing You'an Hospital</i>			
Exhaustion Panel-1			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD
CTLA-4	PE-cy7	349914	Biolegend
Tim3	BV421	345008	Biolegend
KLRG-1	BV605	138419	Biolegend
2B4	APC	329512	Biolegend
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 2

<i>Panel acted from August 2014 to July 2015 at Beijing You'an Hospital</i>			
Exhaustion Panel-2			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-cy7	560179	BD
CD160	PE-cy7	341212	Biolegend
BTLA (CD272)	APC	344510	Biolegend
PD-1	BV650	564104	BD
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 3

<i>Panel acted from August 2014 to July 2015 at Beijing You'an Hospital</i>			
Differentiation Panel			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD
CD27	PE-cy7	356412	Biolegend
CD45RA	BV421	304130	Biolegend
CCR7	BV605	353224	Biolegend
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 4

<i>Panel acted from August 2015 to July 2016 at Beijing You'an Hospital</i>			
Exhaustion Panel			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD
CTLA-4	PE-cy7	349914	Biolegend

TGIT	PE	12-9500-42	eBioscience
KLRG-1	BV605	138419	Biolegend
2B4	APC	329512	Biolegend
PD-1	BV650	564104	BD
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 5

<i>Panel acted from August 2015 to July 2016 at Beijing You'an Hospital</i>			
Differentiation Panel			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD
CD27	PE-cy7	356412	Biolegend
CD45RA	BV421	304130	Biolegend
CCR7	BV605	353224	Biolegend
2B4	APC	329512	Biolegend
PD-1	BV650	564104	BD
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 6

<i>Panel acted from 17/01/2016 to date at Beijing You'an Hospital</i>			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD
CTLA-4	PE-Cy7	349914	Biolegend
Tim3	BV421	345008	Biolegend
KLRG-1	BV605	138419	Biolegend
2B4	APC	329512	Biolegend
PD-1	BV650	564104	BD
TIGIT	PE	12-9500-42	eBioscience
CD27	PERCP5.5	356407	Biolegend
CD45RA	BV785	304139	Biolegend
CCR7	BV711	353227	Biolegend
CD56	PE-Dazzle594	318347	Biolegend
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 7

<i>Panel acted from August 2015 to January 2016 at Xinjiang Cancer Hospital</i>			
Exhaustion Panel-1			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD

CTLA-4	PE-cy7	349914	Biolegend
Tim3	BV421	345008	Biolegend
KLRG-1	BV605	138419	Biolegend
2B4	APC	329512	Biolegend
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 8

<i>Panel acted from August 2015 to January 2016 at Xinjiang Cancer Hospital</i>			
Exhaustion Panel-2			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-cy7	560179	BD
CD160	PE-cy7	341212	Biolegend
BTLA (CD272)	APC	344510	Biolegend
PD-1	BV650	564104	BD
Aqua L/D staining	BV510	L34957	Life Technologies

Panel 9

<i>Panel acted from 01/08/2016 to date at Xinjiang Cancer Hospital</i>			
Exhaustion panel			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD
CTLA-4	PE-cy7	349914	Biolegend
Tim3	BV421	345008	Biolegend
KLRG-1	BV605	138419	Biolegend
2B4	APC	329512	Biolegend
TIGIT	PE	12-9500-42	eBioscience
PD-1	BV650	564104	BD
CD56	PE-dazzle 594	318347	Biolegend
L/D staining	BV510	L34957	Life Technologies

Panel 10

<i>Panel acted from 01/08/2016 to date at Xinjiang Cancer Hospital</i>			
Differentiation panel			
Marker	Fluorophore	Catalog No.	Manufacturer
CD3	Alexa Fluor 700	344822	Biolegend
CD4	FITC	345768	BD
CD8	APC-H7	560179	BD
CD27	PE-cy7	356412	Biolegend
CD45RA	BV421	304130	Biolegend
CCR7	BV605	353224	Biolegend

PD-1	BV650	564104	BD
KLRG-1	PE	138408	Biolegend
CD56	PE-dazzle 594	318347	Biolegend
L/D staining	BV510	L34957	Life Technologies

2.4.2 Applying FMO control in the gating of inhibitory receptors in multichromatic flow cytometry

The revolution of flow cytometry techniques allow researchers to apply multi-colors staining in one experimental tube, substantially reducing the minimum required cells obtained for human samples and minimizing time spent in data acquisition. However, the multichromatic panels need advanced techniques to adjust the compensation and control data qualities. To test our research questions we have conducted up to 14-color flow cytometric panels to simultaneously investigate the expression of inhibitory receptors on T cells and its correlation within distinctive T-cell differentiation stages in both PBMCs and TILs from multiple cancer patients. Previous studies have indicated that most of the inhibitory receptors did not exhibit a clear separation in dot pots, which may potentially introduce biases and mask the differential expression of a certain marker among different disease stages or between healthy donors and cancer individuals. Hereby, a quality control and accurate gating strategy should be performed at a regular base. We routinely conducted FMO (Fluorescence Minus One) control to precisely gate on the positive population of inhibitory receptors or T-cell differentiation markers and to control the quality of our *ex vivo* phenotypic analysis. FMO is the control staining of multichromatic flow cytometry panel by not adding one antibody into the staining cocktail in order to observe the fluorescent spill-over into that channel. More importantly, it also

serves as an important tool for monitoring machine performance and detecting potential instabilities from hardware.

Specifically our FMO control was performed at biweekly frequency in both PBMCs and TILs. Machine status was daily checked by CS&T beads. All the antibodies were titrated in Oxford lab before transporting to China. Based on each antibody's titration, the FMO cocktails were made and listed in the following chart (Table 2.3). Gating examples were demonstrated in Figure 2.2. As CD3, CD4 and CD8 are well separated in the dot plots. All the FMO tubes would add CD3, CD4 and CD8 antibodies. Eventually together with full color staining, all the markers of inhibitory receptors and T-cell differentiation markers could be gated accurately.

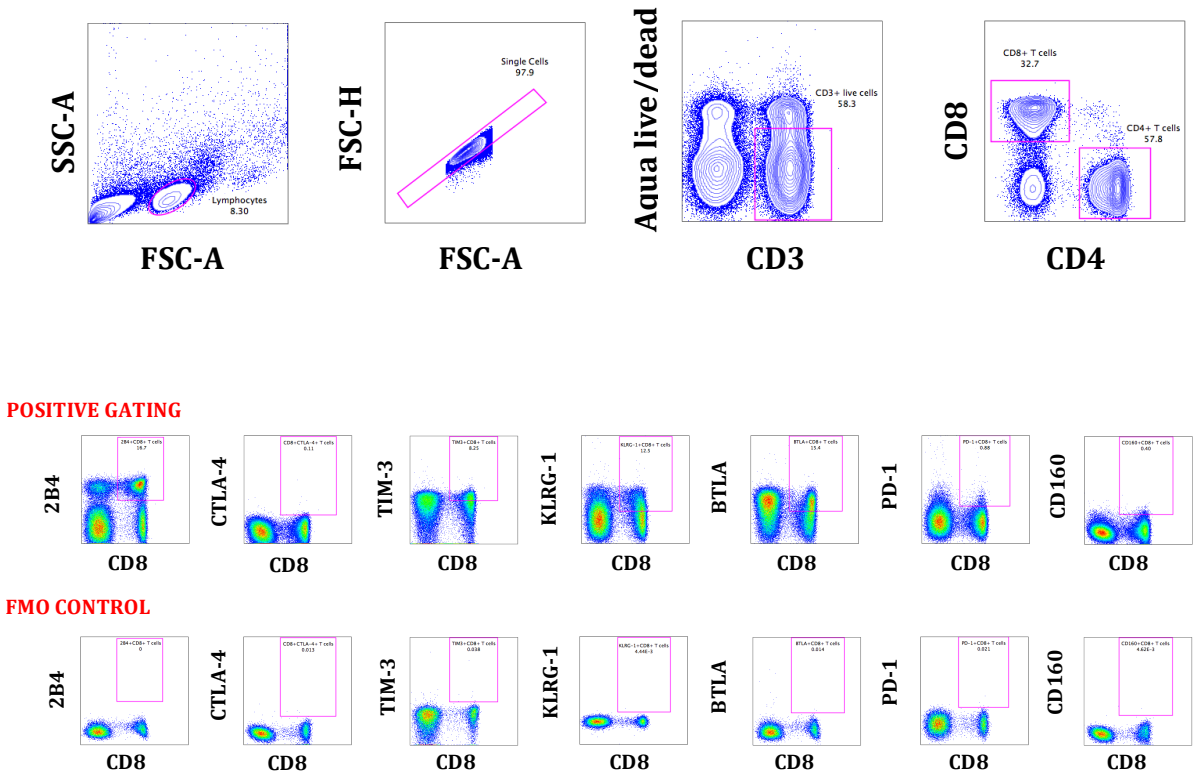
In addition, the FMO control also serves as a sensitive method to monitor machine's performance. The voltages of each lasers naturally fluctuated, which in turn may affect cell population's position in dot plots. Normally all the clinical samples received within a long period (more than 1 year) in which the position of positive population of a certain inhibitory receptor could be drifted to 10^1 to 10^2 in dot plots on the same machine. In order to compare all the data acquired in different time, we apply FMO control within 2 weeks of data acquisition time as the standard to gate the frequencies of inhibitory receptors.

Table2.3: FMO control chart

PE- Dazzle594	BV711	BV785	PERCP5.5	PE	BV650	APC	BV605	BV421	PE-Cy7	Fluorophore
CD56	CCR7	CD45RA	CD27	TIGIT	PD-1	2B4	KLRG-1	Tim-3	---	CTLA-4 FMO
CD56	CCR7	CD45RA	CD27	TIGIT	PD-1	2B4	KLRG-1	---	CTLA-4	Tim3 FMO
CD56	CCR7	CD45RA	CD27	TIGIT	PD-1	2B4	---	Tim-3	CTLA-4	KLRG-1 FMO
CD56	CCR7	CD45RA	CD27	TIGIT	PD-1	---	KLRG-1	Tim-3	CTLA-4	2B4 FMO
CD56	CCR7	CD45RA	CD27	TIGIT	---	2B4	KLRG-1	Tim-3	CTLA-4	PD-1 FMO
CD56	CCR7	CD45RA	CD27	---	PD-1	2B4	KLRG-1	Tim-3	CTLA-4	TIGIT FMO
CD56	CCR7	CD45RA	---	TIGIT	PD-1	2B4	KLRG-1	Tim-3	CTLA-4	CD27 FMO
CD56	CCR7	---	CD27	TIGIT	PD-1	2B4	KLRG-1	Tim-3	CTLA-4	CD45RA FMO
CD56	---	CD45RA	CD27	TIGIT	PD-1	2B4	KLRG-1	Tim-3	CTLA-4	CCR7 FMO
---	CCR7	CD45RA	CD27	TIGIT	PD-1	2B4	KLRG-1	Tim-3	CTLA-4	CD56 FMO

Figure 2.2:

A: FMO controls of 7 inhibitory receptors to facilitate gating the position of positive population on CD8 T cells



B: FMO controls of 3 differentiation markers to facilitate gating the position of positive population on CD8 T cells

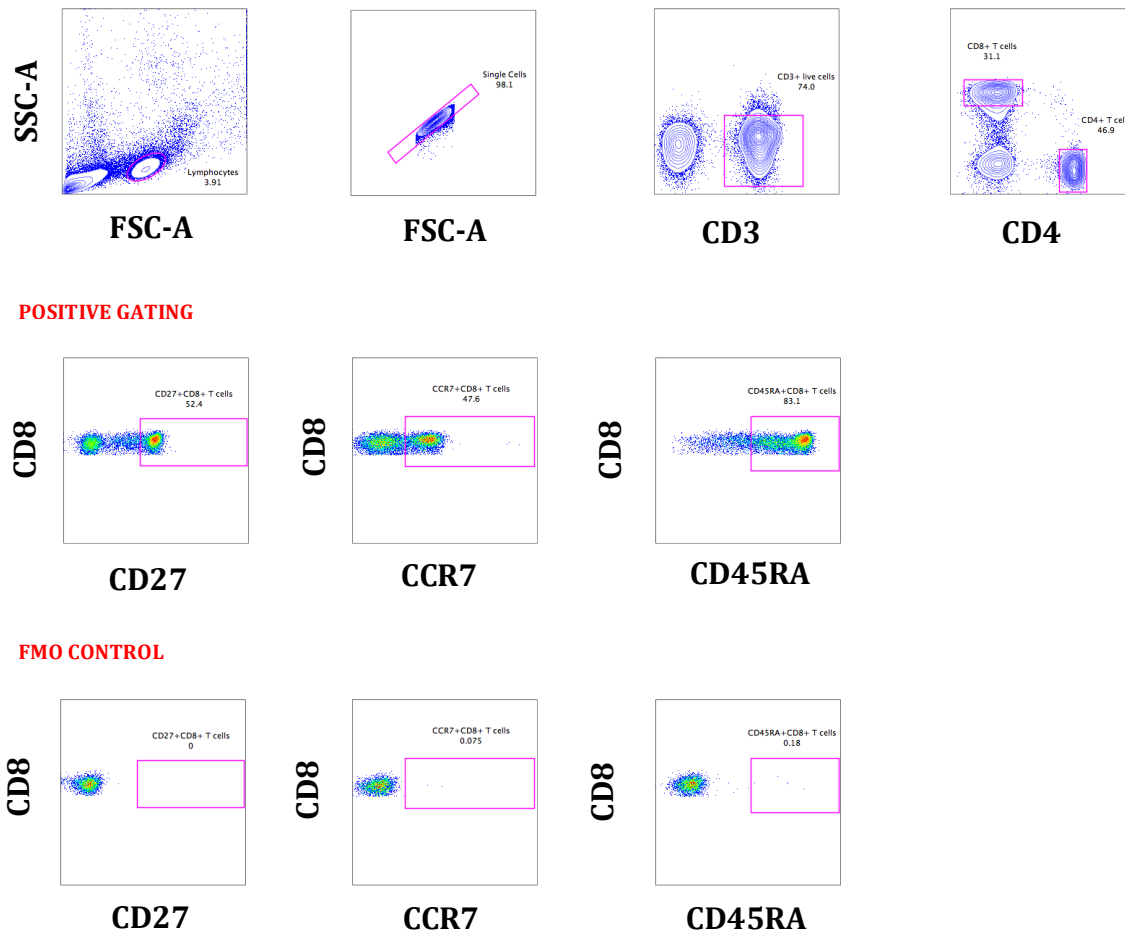


Figure 2-2: Gating strategy example from PBMCs of a breast cancer individual; Progressive gating strategy used to quantify the expression of 7 inhibitory receptors (Figure 2-2-A) and 3 differentiation markers (Figure 2-2-B) on CD4+ T cells and CD8+ T cells from fresh PBMCs in breast cancer individuals; FMO control was applied to localize the gating position of sole inhibitory receptor on T cells; all FMO tubes were stained with live/dead staining and anti-CD3, anti-CD4 and anti-CD8 before the staining of FMO cocktails. Apart from 2B4, the rest 6 inhibitory receptors do not have visually clear separated population to distinguish positive and negative subsets in dot plots. Hence, FMO control is a useful tool to accurately gate inhibitory receptors on T cells.

2.5 Paraffin embedded human tissues treatment and staining

Paraffin embedded human tissue blocks were kept in room temperature and transferred to Pathology department in our collaborative hospitals. Before experiments, blocks were cut to 10 micron per slice at Pathology Department in Xinjiang Cancer Hospital and Oxford Histopathology Research Centre in John Radcliffe Hospital. 10 slices were obtained from each block. To control the biases, all the blocks for our experiments were sliced at same time. Slides were kept at room temperature without light exposure.

2.5.1 De-waxing of paraffin embedded sections

The slides were de-waxed firstly in HistoClear solution (Agar Scientific, HS-200) for 5 minutes twice. Then it was immediately transferred to 100% ethanol for 5 minutes twice. Hereafter, slides were immersed in 75% ethanol for 5 minutes twice and followed by 50% ethanol for 5 minutes twice. Finally slides were washed in distill water for 10 minutes to remove all the reagents left on the slides.

2.5.2 Antigen retrieval of paraffin embedded sections

Paraffin embedded technique has stored numerous human samples for medical researches and is convenient for transportation. It has been reported that no significant gene expression profiling was observed between the 10 years finely stored blocks and newly stored blocks from liver cancer surgical samples.

Normally formalin-fixed tissues require an antigen retrieval procedure before

immunohistochemical staining. Because methylene bridges were formed during fixation cross-link proteins and it could mask antigenic sites for binding detecting antibodies. The purpose of antigen retrieval methods is to break these methylene bridges and expose antigenic sites for detection. Currently 2 methods for antigen retrieval are widely applied: heat induced epitope retrieval (HIER) and enzymatic retrieval. Pure enzymatic retrieval may damage morphologic features of the section, which would lose valuable information especially in our experiments. Therefore, in our optimization of staining 3 ligands (PD-L1, e-cadherin and Galectin-9) in human tumor samples, we have optimized the protocols to maximize the exposure of antigen while maintaining the structure of samples to the best extent.

Glass dishes were loaded with $\frac{1}{2}$ to $\frac{3}{4}$ tank 1X Target Retrieval Solution (DAKO, Lot: 10110578). 1X Target Retrieval Solution was diluted from 10X Target Retrieval Solution by distilled water before experiments. It was reusable with 2 months and kept at room temperature avoiding lights. Then glass dishes were pre-heated in the microwave for 5 minutes at 800W till boiling covered by foil on top. Later, all the slides were gently transferred to the glass dishes covered by foil on top, which were subsequently heated in the microwave for 20 minutes at 150W. All the slides were removed from the glass dishes afterwards to the dishes containing room temperature PBS for 10 minutes. Tissue papers were used to dry the redundant PBS on the slides and transferred into staining tray covered by the lid (Thermo Fisher, Lot: 21053).

2.5.3 Treatment for autofluorescence reduction in paraffin embedded human tissues

During our optimization of ligands expression analysis on human tumor paraffin embedded samples we have noticed significant autofluorescence from paraffin embedded liver cancer samples and breast cancer samples. Other studies also reported the autofluorescence was commonly detected in both human and murine renal tissues, lung tissues and spleen tissues[190, 191]. Several factors contribute to the auto-fluorescence of human paraffin embedded samples. 1: human tissues consist of natural substances such as flavins and porphyrin in live human tissues that could generate the natural “background” in the excitation of lasers; 2: Under excitation from 360nm to 647nm wavelength from lasers, strong auto-fluorescence caused by a common products-lipofuscins that could be detected in a wide range of cell types, especially in vessel rich organs such as liver, kidney and spleens. 3: Reagents used in the sample fixation and antigen retrieval could cause auto-fluorescence such as aldehyde. Reducing autofluorescence is the prerequisite for the quantification of ligand expression in tumor microenvironments. Hereby, we have applied multiple methods including sodium borohydride treatment (Sigma Aldrich, CAS Number: 16940-66-2), TrueBlack quencher (Biotium, Catalog No. 23007) treatment and Sudan Black (Sigma Aldrich, CAS Number: 4197-25-5) treatment, among which TrueBlack quencher treatment has the best effects in reducing autofluorescence while maintaining the morphologic structure of the tissues. Details are as follows.

After antigen retrieval, just before use, dilute 20X TrueBlack solution to 1X in 70% ethanol. Immediately dropping 1X TrueBlack solution onto slides and covered by lid to prevent evaporation. Waiting 1 minute or more, if the TrueBlack solution is nearly dry, add more drops on to the slides. Transferring the slides to glass dishes and wash slides with PBS 3 times. Then use tissue papers to dry out the redundant PBS on the slides.

2.5.4 Fluorescent staining in human paraffin embedded sections

In our ligands expression analysis, we in total investigated 3 ligands expression, PD-L1, e-cadherin and Galectin-9. Having optimized several primary antibodies of 3 ligands and referring to other studies, we have finally chosen anti-PD-L1 (Abcam, catalog: ab205921), anti-e-cadherin (Abcam, catalog: ab1416) and anti-Galectin-9 (Abcam, catalog: ab69630) for our experiments. Secondary antibodies are AbberiorRed goat anti-rabbit (Abberior, catalog No. 2-0012-011-9) and AbberiorRed goat anti-mouse (Abberior, catalog No. 2-0012-011-2) respectively. Before primary antibody staining, slides were blocked with 5% Normal serum solution for 30 minutes to limit non-specific binding (5% goat serum into sterile PBS). Then slides were stained by 150ul primary antibody working solution (primary antibody 1:50 diluted into 5% normal serum solution) overnight at 4 degrees Celsius without exposure to lights. Next day, slides were washed by PBS for 5 minutes in glass dishes. Then it was stained by 150ul secondary antibody working solution (secondary antibody 1:250 diluted into 5% normal serum solution) for 1 hour. After staining, slides were washed in PBS and dried by tissue papers. Slides were then mounted by mounting medium and covered by slips.

2.6 Quantification of ligands expression by confocal microscopy

After staining, all the slides were loaded on Zeiss 880 confocal microscopy for the detection and quantification of ligands expression. Due to the autofluorescence in green channel (488nm) and blue channel (561nm), we only use the far red channel (633nm) for 3 ligands expression analysis. 1 drop of Immersol oil (Zeiss, catalog: 120711) was added onto each slide, Then slides were loaded onto the tray inserted in the microscopy. Objective lens is 25X/0.8 in oil mode. Dimension is 1024X104 with 12 bit depth. Laser power of far red for the detection of e-cadherin is 16 units with 807 laser gain. In the meanwhile laser power of far red for the detection of PD-L1 and Galectin-9 is 5.5 units with 807 laser gain. For quantification analysis images were randomly collected 4 times per slide and mean of the expression was calculated. Negative control slide was treated as same as full staining but lack of primary antibody staining. Images of green channel and blue channel were also acquired as references.

To control the studies and in order to yield less biased data, slides from 39 breast cancer individuals and 7 breast fibroadenoma individuals were stained in same day and analyzed on confocal microscopy within a week. Then all the data exported to FIJI software for fluorescent intensity analysis.

2.7 Data analysis

2.7.1 Data analysis of the expression of inhibitory receptors on T cells

Expression of 7 sole inhibitory receptors on T cells and expression of 6 inhibitory receptors on NK cells or NK T cells were plotted in Prism 7 software and analyzed by ordinary one-way ANOVA test. False discovery rate in multiple t tests was controlled by two-stage step-up method of Benjamini, Krieger and Yekutieli. In non-multiple t tests, non-parametric Mann-Witney t test was applied to compare the frequencies of inhibitory receptors on T cells.

2.7.2 Data analysis of correlation between the expressions of inhibitory receptor and ligand

Pearson correlation coefficient (Pearson's r) was applied to investigate correlation of expression between PD-1/PD-L1, KLRG-1/e-cadherin and Tim-3/galectin-9. R squared value and P value were calculated and plotted inside Prism7 software.

2.7.3 Big data analysis in FIJI software with R language

Ligands expression of paraffin embedded samples from 39 breast cancer and 7 breast fibroadenoma individuals were fluorescently quantified in FIJI software with codes written in R language to formalize all the images and yield comparable fluorescent intensity data.

2.7.4 High-throughput bioinformatics in single cell analysis

In this thesis, SPADE analysis, viSNE analysis and CITRUS analysis were applied to reveal the heterogeneity of lymphocytes in HBV chronically HBV infected individuals and HBV associated HCC individuals. They are all algorithm based. The core theory is to reduce the high dimensionalities to 2 dimension plots while maintaining cell-cell distance. SPADE and viSNE concentrated on marker expression of individual at single cell level whereas, CITRUS analysis investigated marker expression in population level.

2.7.4.1 SPADE analysis

SPADE is a genuine visualization tool that was initially designed to organize heterogeneous populations of single-cell data onto a 2D tree representation based on the similarities of a group of cells under selected markers in unsupervised manner. The nodes of the SPADE tree represent clusters of groups of cells that share similar marker expression. SPADE uses the size and color of each node to represent the number of cells and median marker expression, respectively, in order enabling users to quickly review a high-dimensional parameter space with a 2D tree display. The branching structure of the tree can be used to infer cellular hierarchies when the tree is built using lineage-related surface markers.

2.7.4.2 viSNE analysis

viSNE is an algorithm based program that reduces high dimensional flow cytometric data into 2-dimensional scatter plot while maintaining the structure

of cells[192]. Its core algorithms were firstly developed in 2008 known as t-Distributed Stochastic Neighbor Embedding (t-SNE) in order to visualize high-parameters data in multiple fields. viSNE was applied to the identification of leukemia cancer cells in human cancerous bone marrow samples at single-cell level through CytoF. The algorithms tested cells through several steps. 1: based on all the parameters in the experimental panel, viSNE calculates the fluorescent intensity of each marker on the cells; 2: then viSNE algorithms run a pair-wise study to compare the similarities of cells contained in the samples based on sole marker expression and co-expression of multiple markers; 3: next, viSNE algorithms rank all the cells obtained from the samples according to its expression of all the markers in the panel; 4: finally, visualization algorithms plot entire population selected into 2 dimensional scatter plot at single-cell level. Hereby, phenotypically similar cells would be aggregated into clouds in viSNE map, while maintaining the “distance” of cells.

2.4.7.3 CITRUS analysis

CITRUS (cluster identification, characterization, and regression) is an algorithm that leverages unsupervised clustering and supervised predictive or correlative statistical association models to call out significant features between experimental groups. In this study, we applied the Significance Analysis of Microarrays (SAM, correlative model) and Nearest Shrunken Centroid (PAMR, predictive model) models to investigate that if any subsets are significantly changed in proportion or in expression of a marker among different stages of chronically HBV infected individuals.

3 Chapter 3: *ex vivo* characterization of inhibitory receptors on T cells from different cancer individuals

3.1 Introduction

In T-cell mediated immune responses to chronic virus infection and cancer share multiple similarities. Firstly, both chronic virus infection and cancer are capable of expressing many proteins that could be recognized by T cells as “foreign antigens” eliciting T-cell mediated immune responses[193]. Secondly, with continuous exposure to those foreign antigens in chronic virus infection or in the cancer microenvironment, it can lead T-cells to a hyporesponsive state by up-regulating the expression of inhibitory receptors on T cells known as T-cell exhaustion[171]. Thirdly, the release of immunosuppressive mediators such as interleukin-10 and TGF-beta by apoptotic cells in chronic virus infection or cancer through pathogen-associated molecular pattern (PAMP) and damage-associated molecular patterns (DAMP), can diminish T-cell responses to virus and cancer[193].

In the scenario of T-cell immunity, co-stimulatory receptors and co-inhibitory receptors on T cells regulate T-cells for effective control of virus and tumors while limiting immunopathology and autoimmunity[68]. However, when this homeostasis is disrupted due to persistent antigen stimulation, T-cells ultimately fail to mediate potent control of virus or cancer[194]. The modulation of the homeostasis of co-stimulatory and co-inhibitory receptors on T cells would be a promising approach to develop the immunotherapy against chronic virus infection and cancer, since the first generation blockade immunotherapy

ipilimumab, a monoclonal antibody blocking CTLA-4, shed a light on the new field of virus and cancer immunotherapy[195]. Further noteworthy benefits have been achieved by blocking the PD-1/PD-L1 inhibitory pathway in multiple cancers, such as melanoma, non-small-cell lung cancer and bladder cancer [72, 78, 84, 196-198]. However, the non-responding ratio of each cancer type varies, indicating different cancer may need different immunoinhibitory pathways to target in order to resuscitating dysfunctional tumor infiltrating T cells and augmenting its anti-tumor responses[97]. With the discovery of numerous additional immunoinhibitory pathways on T cells, more blockade options would be available. Given the range of potential immunoinhibitory pathways in a tumor microenvironment apart from PD-1, the next critical question to tackle is to decide which immunoinhibitory pathway should be targeted in a specific type of cancer and which combinations of immunoinhibitory pathway blockade regimens should be developed to enhance and sustain the anti-tumor responses.

With access to surgical samples, we have had an opportunity to investigate the *ex vivo* expression of inhibitory receptors on T cells from HBV chronically infected hepatocellular carcinoma individuals, breast cancer individuals, colorectal cancer individuals, gastric cancer individuals, esophageal cancer individuals, lung cancer individuals, thyroid cancer individuals and renal cancer individuals. In this chapter we aim to:

- (1) Identify the correlation between the expression of inhibitory receptors on T cells from peripheral blood and tumor tissues of different cancer individuals;
- (2) Dissect the expression of inhibitory receptors on T cells from different tumor microenvironment;

(3) Characterize the co-expression pattern of inhibitory receptors on T cells in different cancers.

3.2 Results

3.2.1 Characterization of sole inhibitory receptor expression on T cells between peripheral blood and tissues

The up-regulation of inhibitory receptors such as PD-1, CTLA-4 and Tim-3 on T cells were initially considered as a consequence of T-cell exhaustion due to persistent antigen stimulation[199, 200]. However, current in vitro functional analysis has shown that some up-regulation of inhibitory receptors may also link to T-cell differentiation and T-cell activation rather than functionalizing as inhibitory pathway exclusively[201]. In addition, organs like livers specifically up-regulate the inhibitory receptors on the intrahepatic T cells to ordinarily limit its responses to avoid autoimmunity as an immunotolerogenic property[68]. We aim to characterize the expression of sole inhibitory receptor expression on T cells between the peripheral and tumor tissues. Specifically this thesis focuses on PD-1, KLRG-1, Tim-3, CTLA-4, BTLA, CD160, 2B4, TIGIT as the inhibitory receptors on T cells that may be potential targets in reinvigorating T-cell exhaustion in chronic HBV virus infection and cancers[29, 70, 114, 161, 202, 203]. we compared paired blood and tumor tissues from HBV associated HCC individuals with that from breast cancer individuals. To control this study, we also recruited and compared the expression of the above-mentioned inhibitory receptors on T cells between peripheral blood and tissues from non-HBV infected benign liver lesion (hepatic hemangioma) individuals and benign breast lesion (fibroadenoma) individuals in this study.

Table 3-1: Clinical information of HBV associated HCC individuals

Total samples:		18
Gender:		Male 78% (14/18) Female 22% (4/18)
Age:		53.667±7.77 years
BCLC Cancer classification:	A	22% (4/18)
	B	28% (5/18)
	C	44% (8/18)
	D	6% (1/18)
TNM Cancer stratification:	TNM Stage I	17% (3/18)
	TNM Stage II	28% (5/18)
	TNM Stage III	55% (10/18)

Table 3-2: Clinical information of HBV negative hepatic hemangioma individuals

Total samples:		8
Gender:		Male 13% (1/8) Female 87%(7/8)
Age:		44.5±10.34 years
Ethnicity:	Han	88% (7/8)
	Uygur	12% (1/8)
	Kazak	(0/8)
	Hui	(0/8)

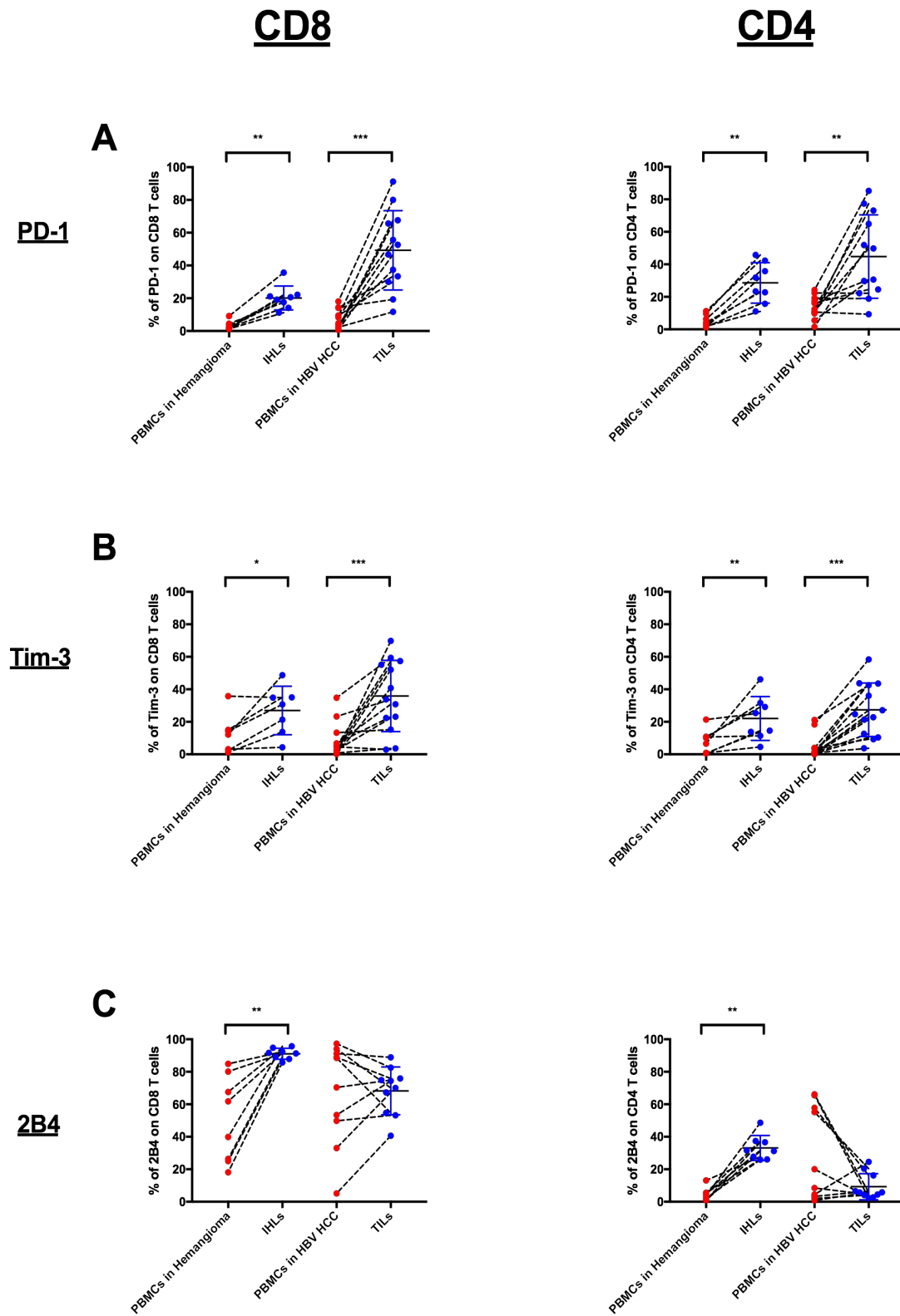
Table 3-3: Clinical information of breast cancer individuals

Total samples:		43
Gender:		Female (100%)
Age:		52.023±10.37 years
Ethnicity:	Han	(29/43)
	Uygur	(10/43)
	Kazak	(3/43)
	Hui	(1/43)
TNM Cancer stratification:	TNM Stage I	23% (10/43)
	TNM Stage II	44% (19/43)
	TNM Stage III	33% (14/43)

Table 3-4: Clinical information of breast fibroadenoma individuals

Total samples:		9
Gender:		Female (100%)
Age:		22±8.02 years
Ethnicity:	Han	100% (9/9)
	Uygur	(0/9)
	Kazak	(0/9)
	Hui	(0/9)

Figure 3-1: Expression analysis of inhibitory receptors on T cells between matched PBMCs and liver tissues in hepatic hemangioma and HBV associated HCC individuals



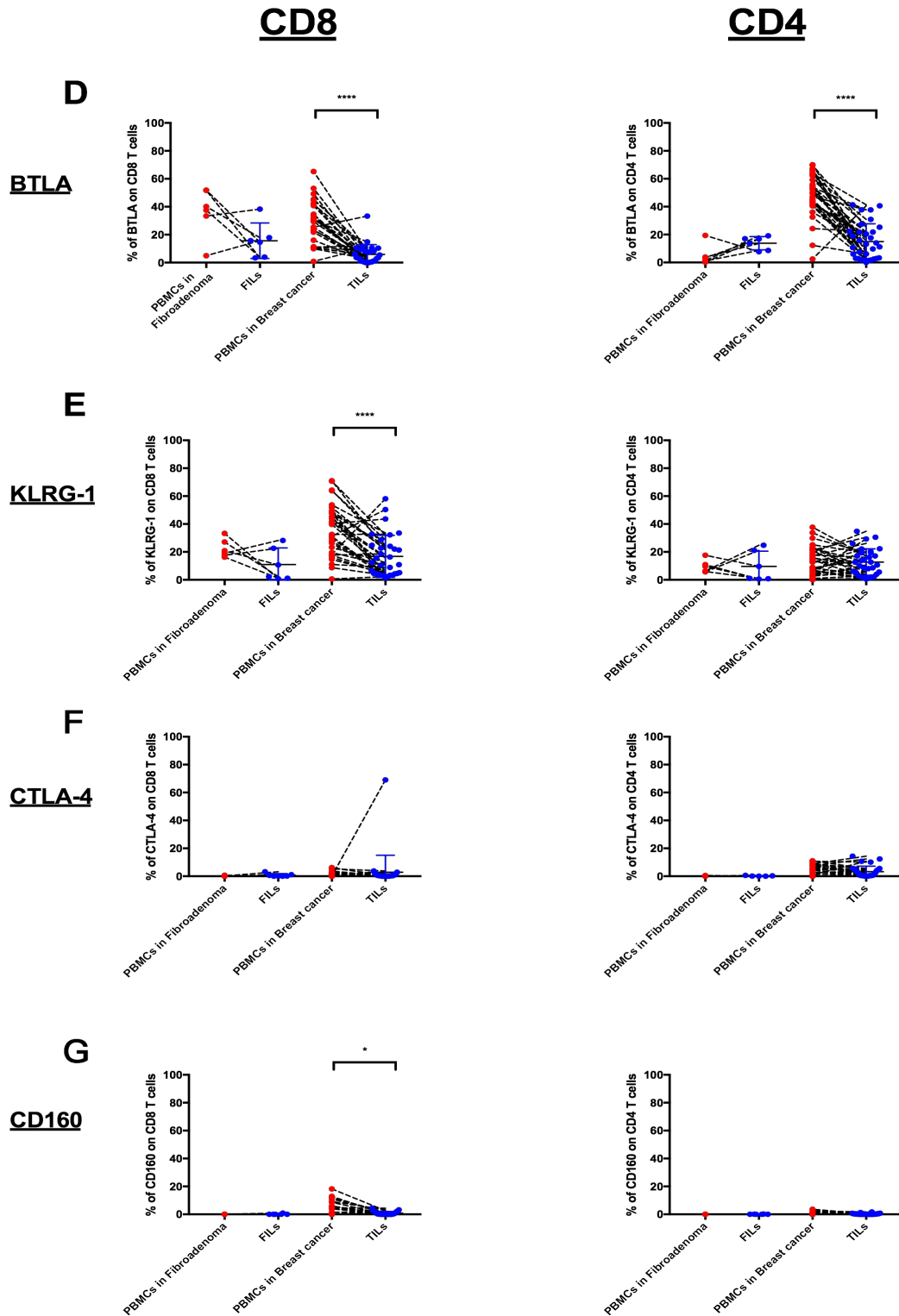
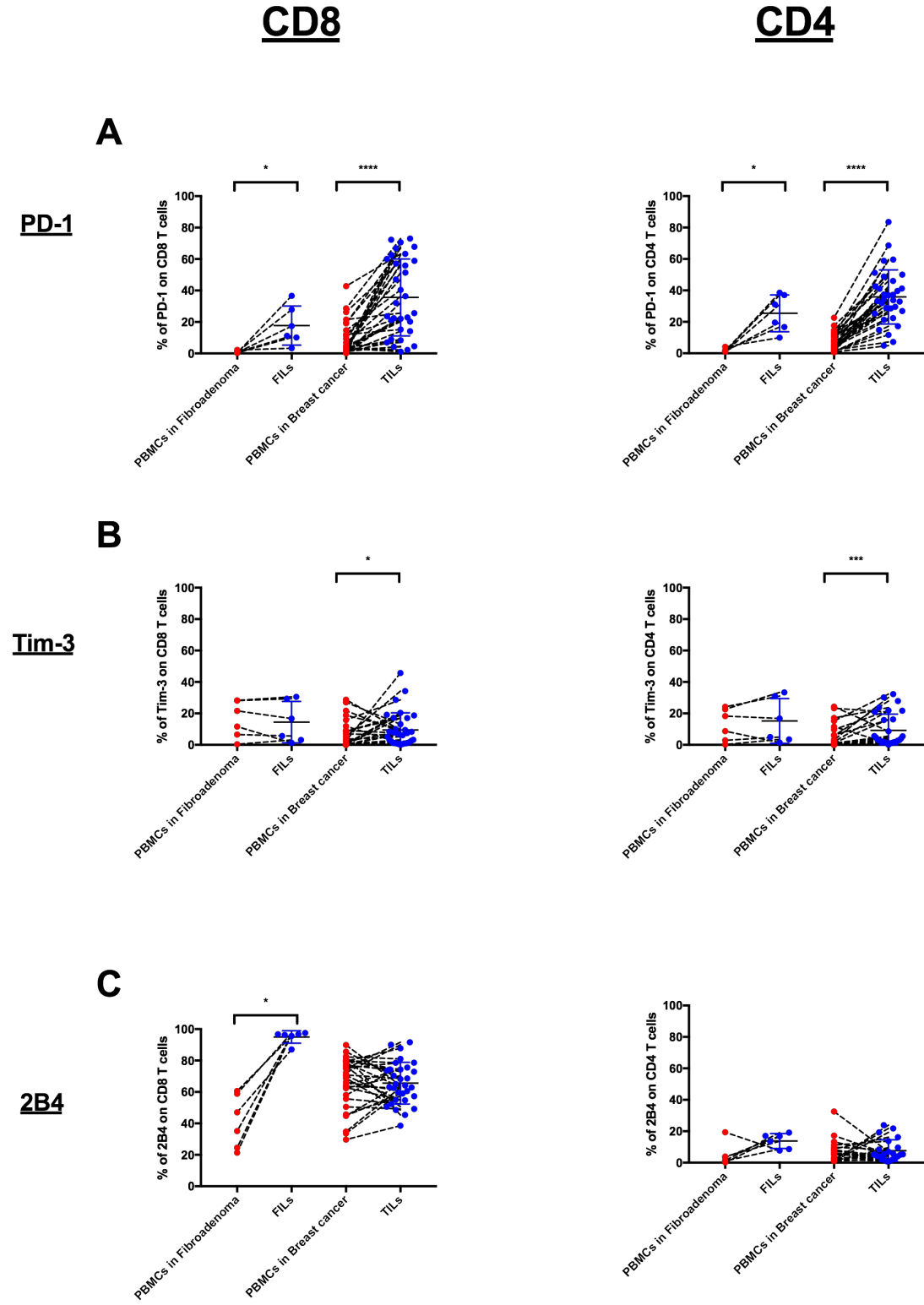


Figure 3-1: Expression analysis of PD-1, TIM-3, BTLA, 2B4, KLRG-1, CTLA-4 and CD160 on CD8+ (two left graphs) and CD4+ T-cells (two right graphs) for patient-matched PBMCs and IHLs or PBMCs and TILs. The pairwise analysis includes 12 HBV associated HCC patients and 8 HBV negative hepatic hemangioma patients (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Paired t-test). **A)** PD-1, **B)** TIM-3, **C)** 2B4, **D)** BLTA, **E)** KLRG-1, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ T cells on PBMCs versus TILs or PBMCs versus IHLs in (%).

Figure 3-2: Expression analysis of inhibitory receptors on T cells between matched PBMCs and liver tissues in breast fibroadenoma and breast cancer



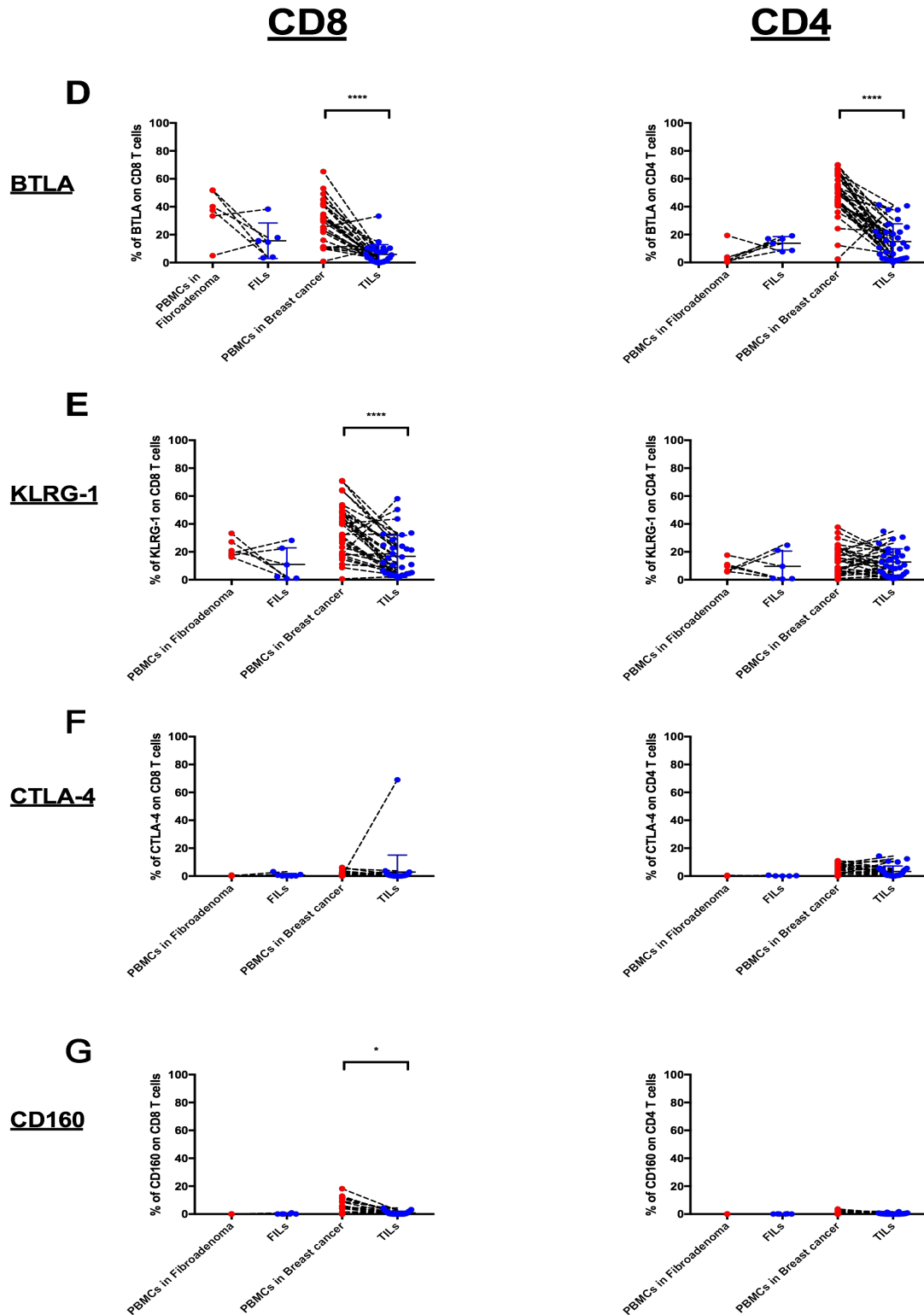
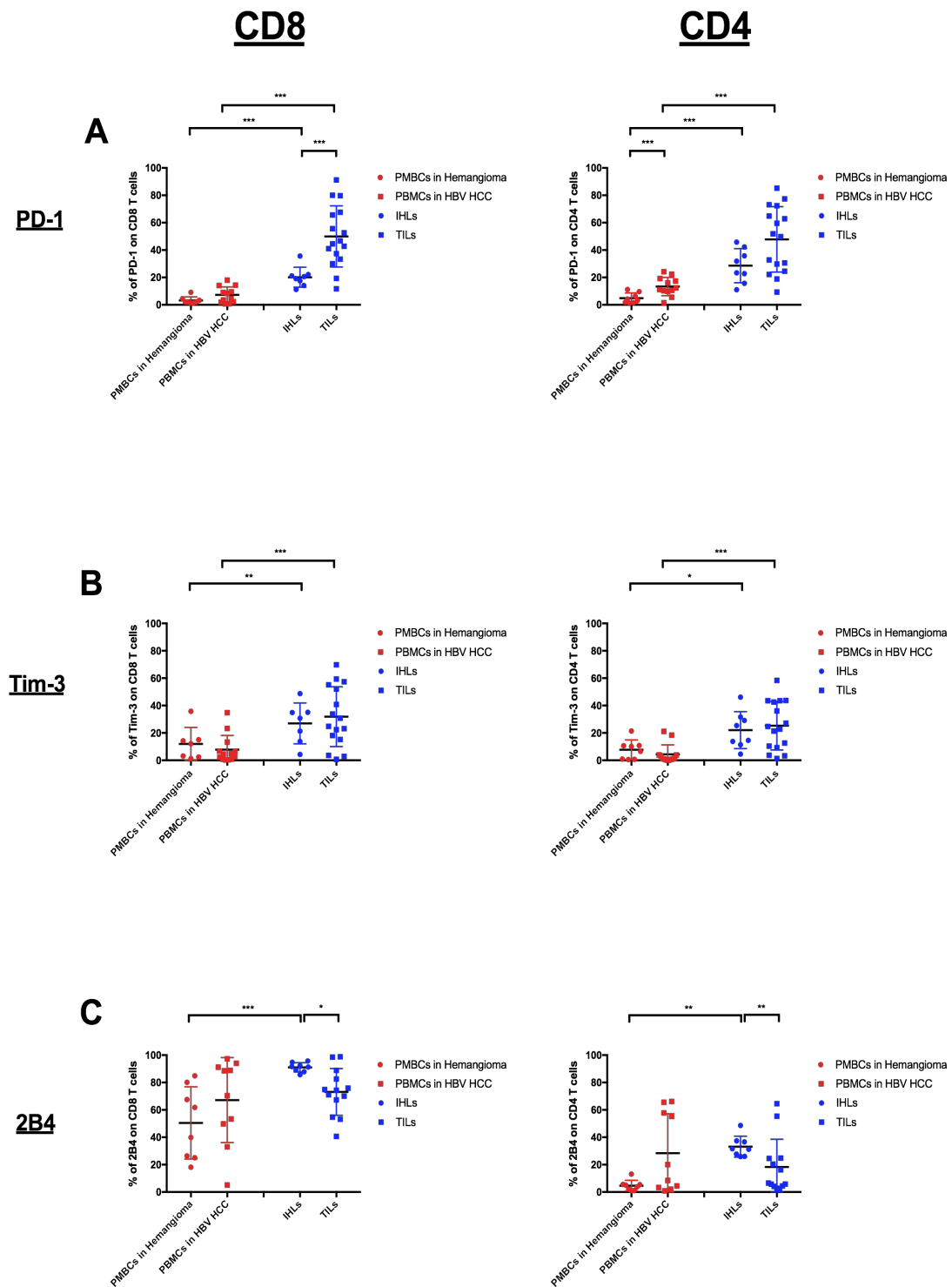


Figure 3-2: Expression analysis of PD-1, TIM-3, BTLA, 2B4, KLRG-1, CTLA-4 and CD160 on CD8+ (two left graphs) and CD4+ T-cells (two right graphs) for patient-matched PBMCs and IHLs or PBMCs and TILs. The pairwise analysis includes 32 breast cancer patients and 6 breast fibroadenoma patients (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Paired t-test). **A)** PD-1, **B)** TIM-3, **C)** 2B4, **D)** BLTA, **E)** KLRG-1, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ T cells on PBMCs versus TILs or PBMCs versus FILs in (%).

Figure 3-3: Expression analysis of inhibitory receptors on T cells between PBMCs and liver tissues



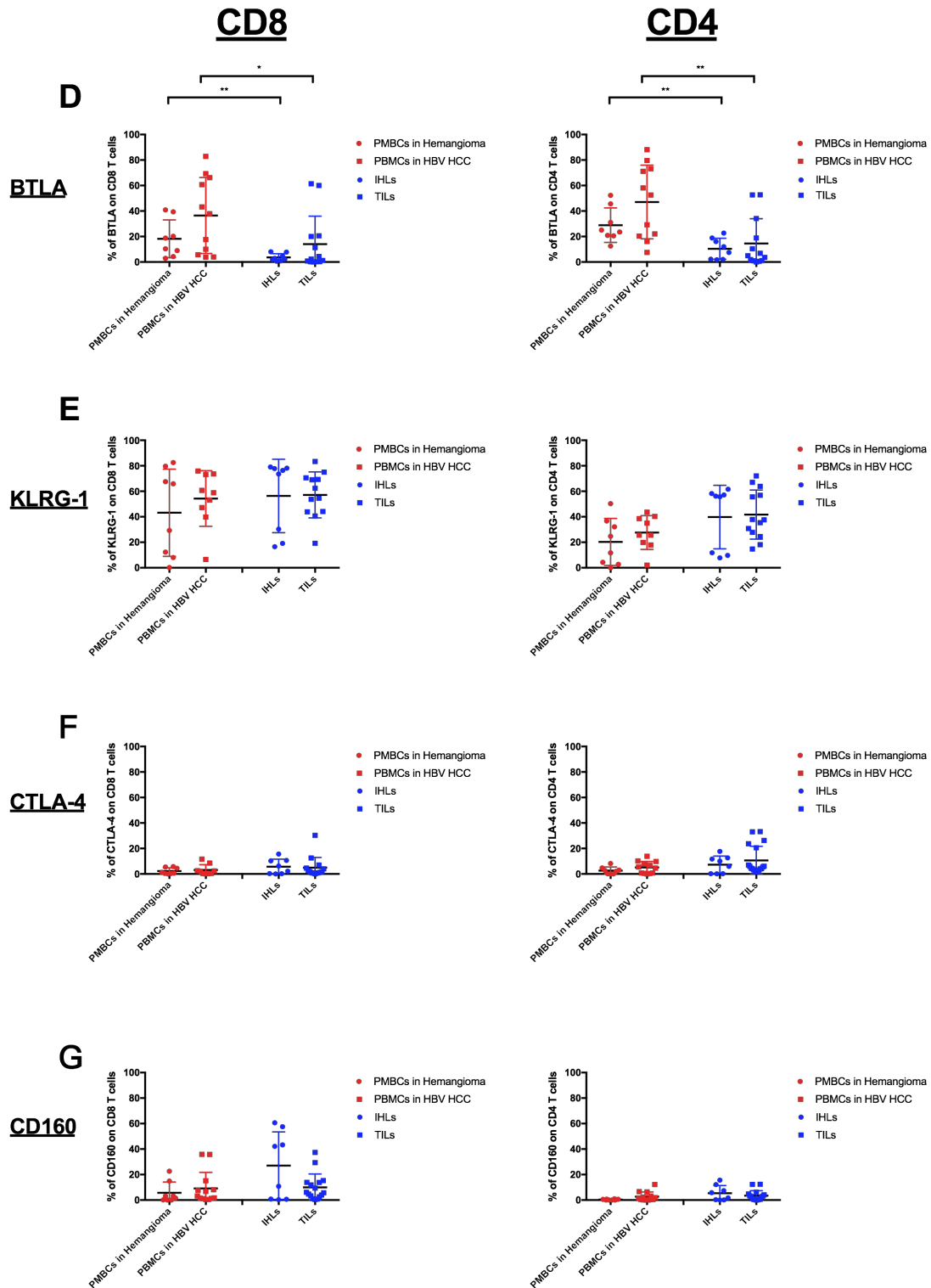
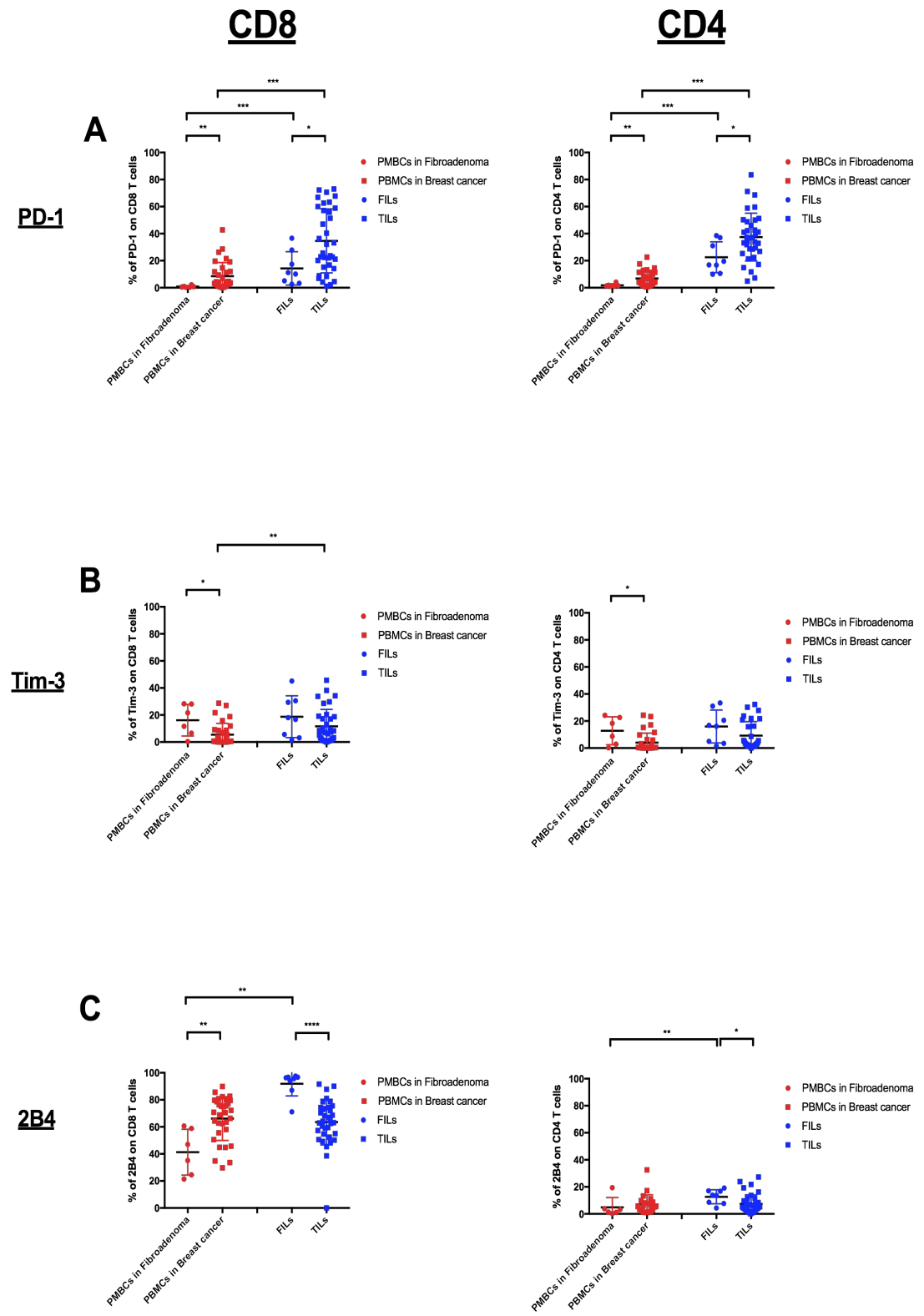


Figure 3-3: Expression analysis of PD-1, TIM-3, BTLA, 2B4, KLRG-1, CTLA-4 and CD160 on CD8+ (two left graphs) and CD4+ T-cells (two right graphs) for non-patient-matched PMBCs and IHLs or PMBCs and TILs. The analysis includes 16 HBV associated HCC patients (12 of which are PMBCs and TILs paired) and 8 paired HBV negative hepatic hemangioma patients (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney t-test). **A)** PD-1, **B)** TIM-3, **C)** 2B4, **D)** BTLA, **E)** KLRG-1, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ T cells on PMBCs versus TILs or PMBCs versus IHLs in (%).

Figure 3-4: Expression analysis of inhibitory receptors on T cells between PBMCs and breast tissues



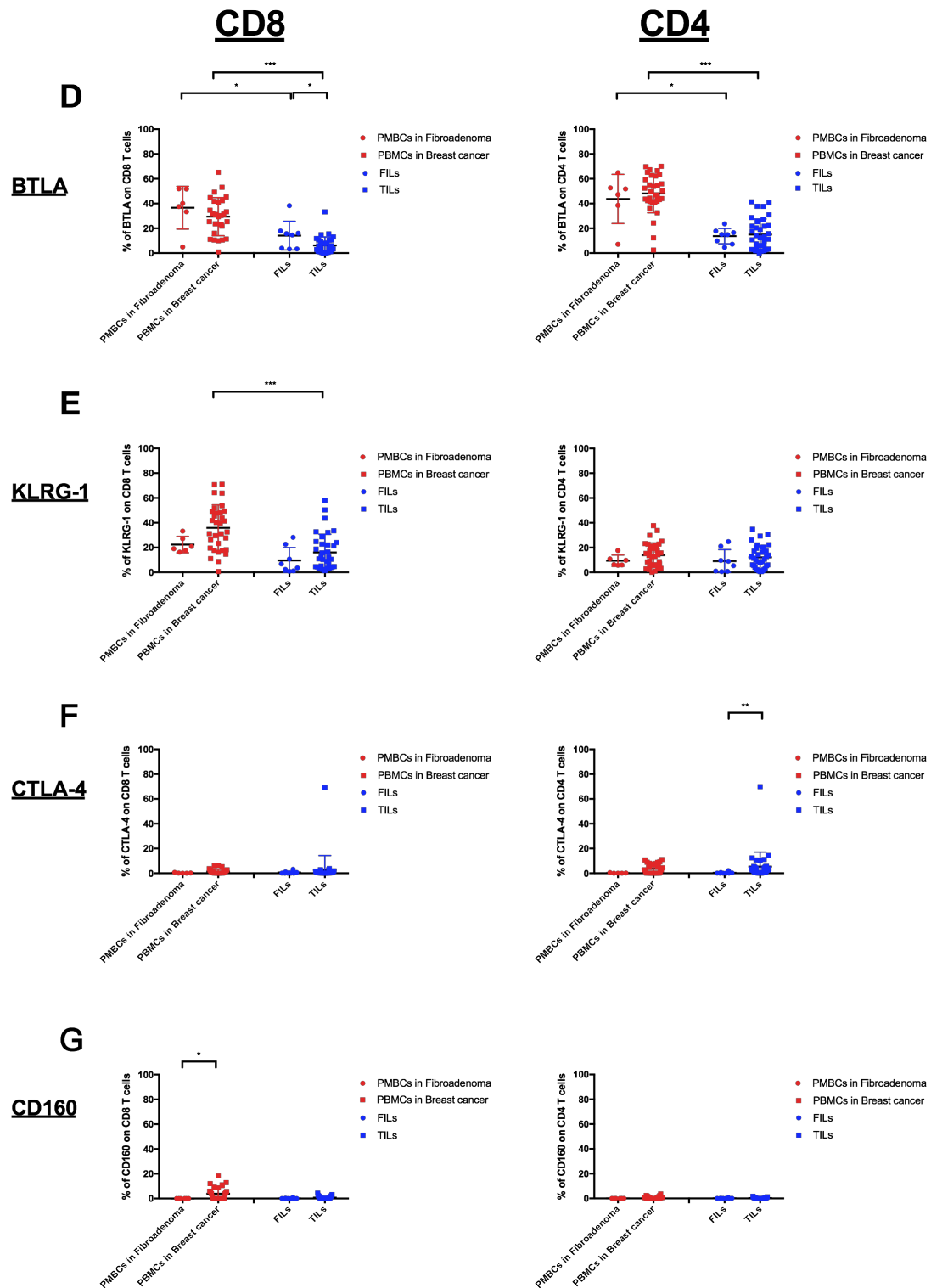


Figure 3-4: Expression analysis of PD-1, TIM-3, BTLA, 2B4, KLRG-1, CTLA-4 and CD160 on CD8+ (two left graphs) and CD4+ T-cells (two right graphs) for non-patient-matched PMBC and TILs or PMBCs and IHLs. The analysis includes 43 breast cancer patients (32 of which are PMBCs and TILs paired) and 9 non-paired breast fibroadenoma patients (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney t-test). **A)** PD-1, **B)** TIM-3, **C)** 2B4, **D)** BTLA, **E)** KLRG-1, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ T cells on PMBCs versus TILs or PMBCs versus IHLs in (%).

In Figure 3-1, the expression analyses of 7 inhibitory receptors on T cells between matched tumor and its counterparts in the periphery were conducted. PD-1 and Tim-3 have a higher expression on both CD4+ and CD8+ TILs compared with its expression in PBMCs from HBV associated HCC individuals. However, the same differential expression levels of PD-1 and Tim-3 on T cells could also be detected in HBV negative benign liver lesions (hepatic hemangioma). Having hypothesized up-regulation of PD-1 and Tim-3 may only be observed in malignant liver tissues as a remarkable phenotype of T-cell exhaustion, this result indicates that higher expression of PD-1 and Tim-3 on bulk TILs in HBV associated HCC individuals do not define T-cell exhaustion.

The higher expression of PD-1 and Tim-3 on T cells from benign liver tissues may associate with self-tolerance as liver is an immunotolerogenic organ in direct contacting with numerous foreign antigens absorbed from the intestines, acting normal physiologic roles[204]. This result is also in line with another study investigating phenotypic significance of inhibitory receptors on T cells from HCV chronically infected individuals when compared with non-HCV benign liver diseases individuals[68]. However, in Figure 3-3, higher expression level of PD-1 and Tim-3 on both CD4 and CD8 TILs were observed when comparing with its expression on IHLs. In parallel, higher expression levels of PD-1 and Tim-3 on both CD4 and CD8 PBMCs from HBV HCC individuals were observed when comparing with its expression on PBMCs from hepatic hemangioma individuals. It indicates the PD-1 and Tim-3 may be the targets in the blockade immunotherapy.

Interestingly, in both HBV HCC individuals and HBV negative benign liver lesion individuals, BTLA has a lower expression on CD4+ and CD8+ T cells in tumor tissues and benign tissues compared with its counterparts in the periphery respectively. It has been reported in others' studies that BTLA has higher expression in naïve T-cells phenotype while decreasing its expression through differentiation[201]. No differential expression of 2B4, CTLA-4 and CD160 on T cells was observed in HBV HCC individuals or HBV negative benign liver lesion individuals. In addition, the global expression of CTLA-4 and CD160 on T cells from either malignant or benign liver tissues is relatively low compared with the expression level of PD-1 and Tim-3. CTLA-4 inhibitor is the first FDA approved drug that achieved promising benefits in treating advanced melanoma patients and has been proposed to have potentials in treating multiple cancers especially combining its usage with conventional cancer treatment regimens such as chemotherapy or radiotherapy.

It is also surprising to observe such a result that CTLA-4 has relatively lower expression (mean expression is 20% of T cells) comparing to PD-1 or Tim-3 expression on TILs from HBV HCC cancer tissues. On the one hand, previous studies indicate that CTLA-4 is predominately expressed on Tregs. The CTLA-4 inhibitor dampens the immunosuppressiveness of Tregs in tumor microenvironment. Hence investigating CTLA-4 expression on conventional CD4 T cells may mask its expression on Tregs. On the other hand, as our data may also suggest that at least in HBV associated liver cancer individuals, CTLA-4 inhibitor may not be a primary choice when considering inhibitory pathway

blockade therapy as its expression on global T cells in tumor microenvironment is relatively low compared to other inhibitory receptors. In addition, CTLA-4 clinical trials in advanced melanoma patients also revealed more severe immune-related side effects than that observed in PD-1 clinical trials.[78]

CTLA-4 may not serve as a preferable inhibitory pathway to block when treating advanced HCC patients especially those with chronic HBV infection. Additional inhibitory pathways involving potent resuscitation of T-cells function with non-severe and manageable side effects would be the goal to prolong overall survival of HBV HCC individuals. Of note, KLRG-1 has a higher expression on both CD4+ and CD8+ T cells from benign liver lesions when compared with its expression in PBMCs. But these expression levels are not observed in HBV HCC individuals. KLRG-1 is an inhibitory receptor on both NK cells and T cells, binding 3 ligands, e-cadherin, v-cadherin and n-cadherin, acting as inhibitory signals[128, 139, 140]. The specific differential expression of KLRG-1 on T cells between the periphery and tumor tissues may indicate its potential in HBV HCC treatment.

Apart from liver cancer, as we had the access to breast cancer and breast fibroadenoma surgical samples, we have also had the opportunity to compare inhibitory receptors expression in other tumor microenvironments. Likewise, in Figure 3-2 the expression analyses of 7 inhibitory receptors on T cells between matched breast cancer tissues and peripheral blood were conducted. PD-1 and

Tim-3 have higher expression on CD4+ and CD8+ TILs compared with its expression on PBMCs from breast cancer individuals. While the same differential expression levels of PD-1 were observed in fibroadenoma individuals but not that of Tim-3. Tim-3 has higher expression on T cells only from malignant tissues rather than benign lesions compared with its expression in PBMCs. In Figure 3-4, higher expression level of PD-1 on both CD4 and CD8 TILs were observed when comparing with its expression on FILs. In parallel, higher expression levels of PD-1 on both CD4 and CD8 PBMCs from breast cancer individuals were observed when comparing with its expression on PBMCs from breast fibroadenoma individuals. Higher Tim-3 expression on CD8 but not CD4 TILs from breast cancer individuals has been found when comparing with its expression on CD8 FILs in breast fibroadenoma. This indicates that in the treatment of advanced breast cancer individuals, PD-1 inhibitor or Tim-3 inhibitor could be considered.

Currently PD-1 inhibitors haven been approved for clinical trials in triple negative breast cancer, a type of poor prognosis breast cancers [55, 205]. Our data indicates that Tim-3 could also be a promising target in breast cancer immunotherapy as it is specifically up-regulated in malignant tissues compared to the periphery. Potentials could be found in a PD-1 and Tim-3 combinatorial blockade regimen of breast cancer treatment, especially in PD-1 blockade non-responders.

Our results are in line with other studies which have shown BLTA has a higher expression in naïve T cells while losing its expression through T cells differentiation[169, 206]. 2B4 has higher expression on T cells in benign breast tissues compared with its expression in PBMCs but this expression pattern of 2B4 has not been found in breast cancer individuals. It indicates that the upregulation of 2B4 on TILs in breast cancer individuals may result from tumor microenvironment linking to T-cell exhaustion. The global expression levels of CTLA-4 and CD160 on T cells in both PBMCs and tissues from breast cancer or fibroadenoma individuals are low although one breast cancer patient has a notably high expression (over 60%) of CTLA-4 on CD4+ and CD8+ TILs. Interestingly, KLRG-1 has a lower expression on CD8+ TILs but not CD4+ TILs compared with its expression in PBMCs from breast cancer individuals. These results imply that CD160 and CTLA-4 may not be the promising targets for blockade immunotherapies in breast cancer individuals. No differential expression of KLRG-1 was found in the comparison between T cells in PBMCs and TILs from fibroadenoma individuals.

With this set of analyses, it enables us to identify differential sole inhibitory receptors expression pattern on TILs between HBV HCC and breast cancer. This may hint that in different cancer, differential sole inhibitory pathway blockade regimen should be considered. The blockade immunotherapy should tailor inhibitory receptor expression on TILs in the tumor microenvironment.

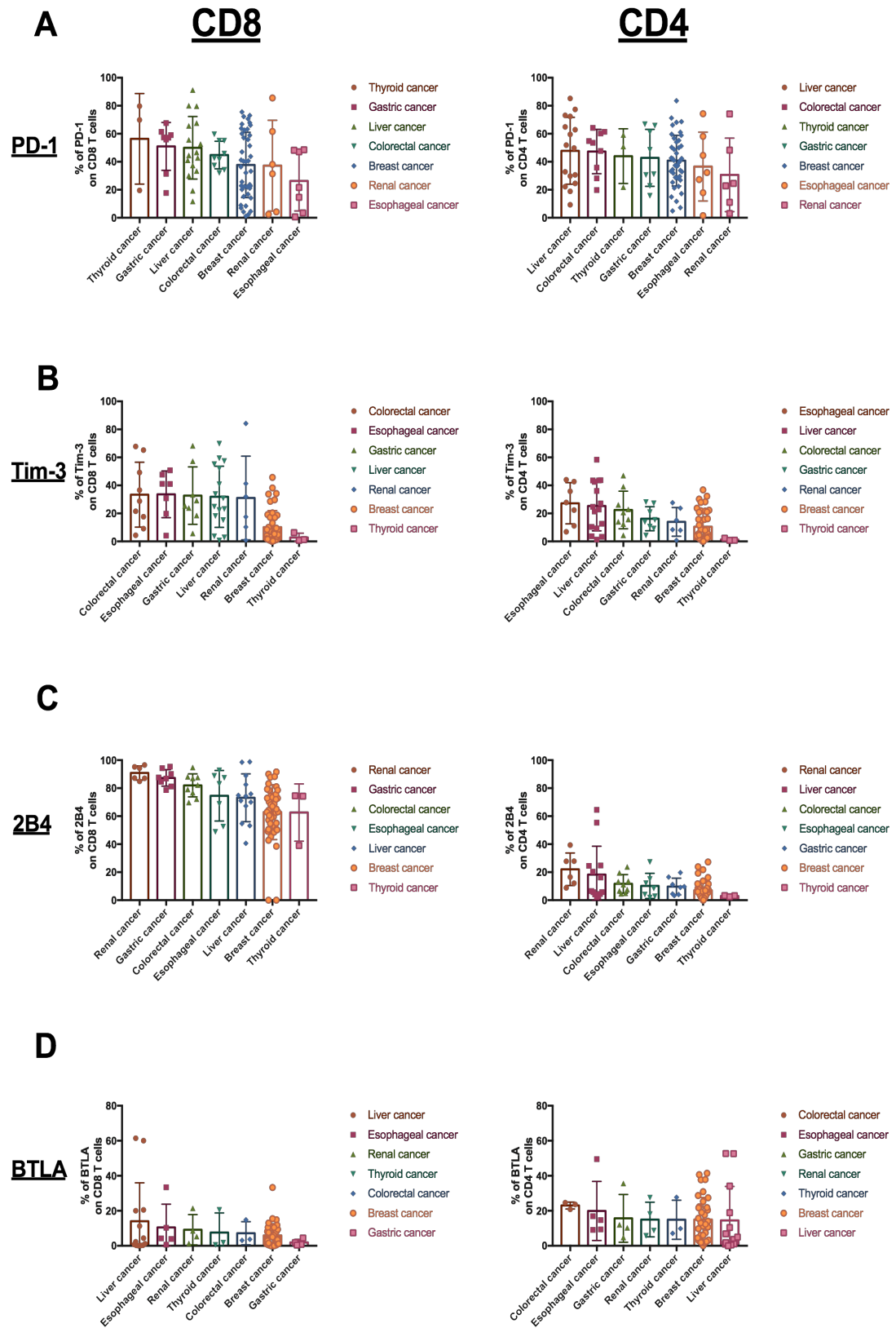
3.2.2 Variable sole inhibitory receptor expression on tumor infiltrating T cells from different cancer individuals

As PD-1/PD-L1 inhibitory pathway monoclonal blockade therapy has delivered inspiring results and sustained anti-tumor benefits for multiple cancers[70, 72, 78, 81, 197]; dissecting the inhibitory pathways in the tumor microenvironment has become the key focus in current cancer immunotherapy[207, 208]. Apart from the heterogeneous identity of primary carcinoma, different carcinomas have differential carcinogenesis mechanisms and foster a unique tumor microenvironment in which the infiltrated immune cells may be altered to different exhausted phenotypes with differential anti-tumor responses leading to different outcomes[209]. Having access to a wide range of surgical samples, we investigated the expression of sole inhibitory receptor on tumor infiltrating T cells from HBV associated liver cancer, breast cancer, gastric cancer, esophageal cancer, colorectal cancer, thyroid cancer and renal cancer. These primary cancers have quite distinctive prognosis, which enables us to compare the *ex vivo* phenotypes of tumor infiltrating T cells residing in different tumor microenvironments. In addition, such an approach may allow us to identify the potential sole inhibitory pathway for blockade immunotherapy to the specific type of cancer.

Table 3-5: Clinical information of primary cancer individuals

Total samples:	94	
Gender:		Male 41% (39/94) Female 59% (55/94)
Age:		52.39±14.29 years
Ethnicity:	Han	73% (69/94)
	Uygur	22% (21/94)
	Kazak	3% (3/94)
	Hui	2% (1/94)
Cancer type:	HBV associated liver cancer	19% (18/94)
	Breast cancer	46% (43/94)
	Esophageal cancer	7% (7/94)
	Gastric cancer	9% (8/94)
	Colorectal cancer	10% (9/94)
	Renal cancer	6% (6/94)
	Thyroid cancer	3% (3/94)

Figure 3-5: Hierarchical expression of inhibitory receptors on tumor infiltrating T cells from different cancers



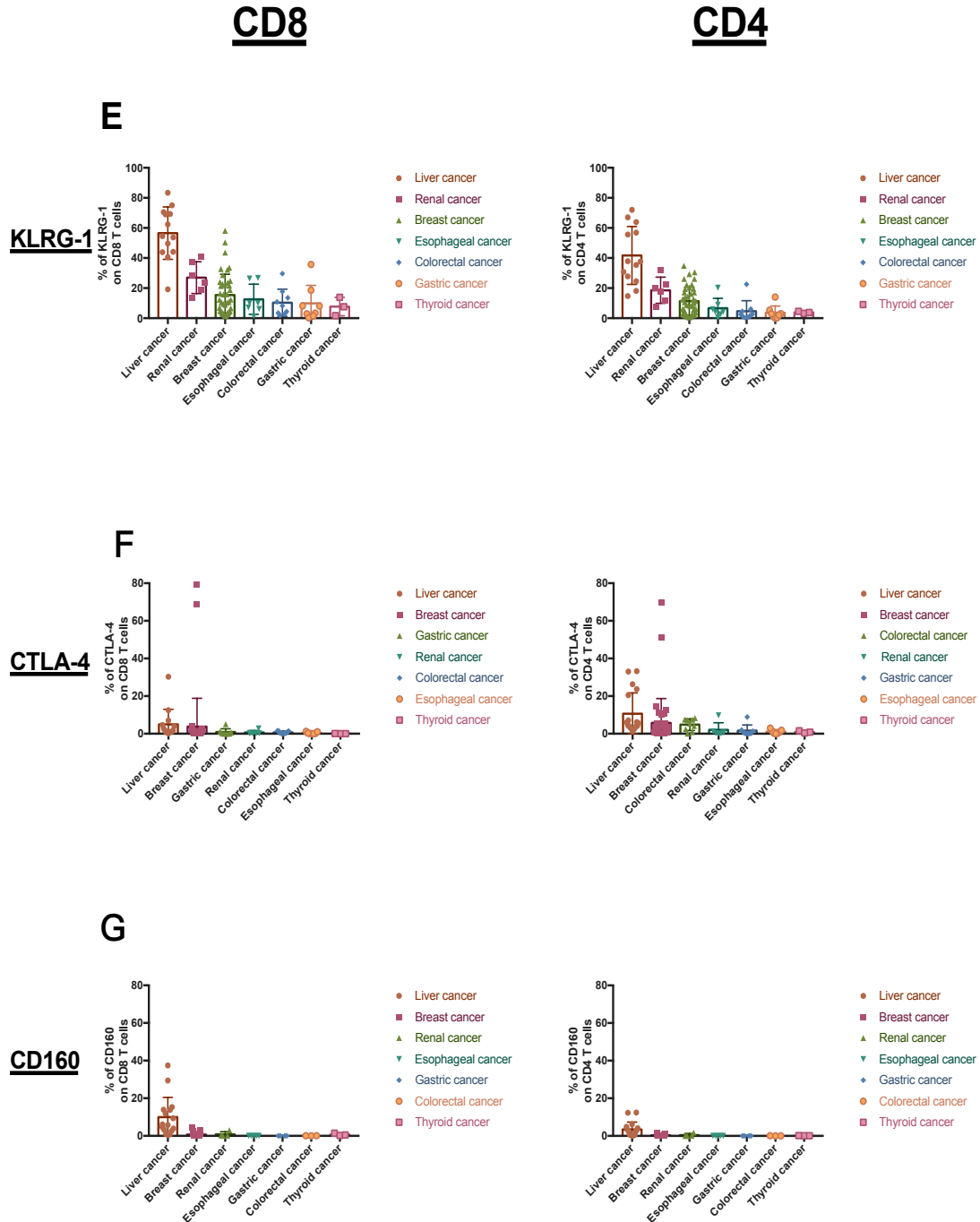


Figure 3-5: *ex vivo* expression analysis of PD-1, TIM-3, BTLA, KLRG-1, 2B4, CTLA-4 and CD160 on CD8+ (left graph) and CD4+ T-cells (right graph) TILs from HBV associated liver cancer, breast cancer, esophageal cancer, gastric cancer, colorectal cancer, renal cancer and thyroid cancer patients. The analysis includes 16 HBV associated HCC patients, 43 breast cancer patients, 6 esophageal cancer patients, 7 gastric cancer patients, 8 colorectal cancer patients, 5 renal cancer patients and 3 thyroid cancer patients. The expression of each inhibitory receptor within different cancers is in hierarchical order. **A)** PD-1, **B)** TIM-3, **C)** 2B4, **D)** BTLA, **E)** KLRG-1, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ TILs in (%).

Table 3-6: Statistics box of Tim-3 expression on CD8 TILs

	Colorectal cancer	Oesophageal cancer	Gastric cancer	Liver cancer	Renal cancer	Breast cancer	Thyroid cancer
Colorectal cancer	NS	NS	NS	*	NS	*	*
Oesophageal cancer	NS	NS	NS	NS	*	*	NS
Gastric cancer	NS	NS	NS	NS	NS	*	*
Liver cancer	*	NS	NS	NS	NS	**	*
Renal cancer	NS	*	NS	NS	NS	*	*
Breast cancer	*	*	*	**	*	NS	NS
Thyroid cancer	*	NS	*	*	*	NS	NS

Table 3-7: Statistics box of Tim-3 expression on CD4 TILs

	Colorectal cancer	Esophageal cancer	Gastric cancer	Liver cancer	Renal cancer	Breast cancer	Thyroid cancer
Colorectal cancer	NS	NS	NS	NS	NS	*	*
Esophageal cancer	NS	NS	NS	NS	NS	*	NS
Gastric cancer	NS	NS	NS	NS	NS	NS	NS
Liver cancer	NS	NS	NS	NS	NS	**	*
Renal cancer	NS	NS	NS	NS	NS	NS	NS
Breast cancer	*	*	NS	**	NS	NS	*
Thyroid cancer	*	NS	NS	*	NS	*	NS

Table 3-8: Statistics box of KLRG-1 expression on CD4 and CD8 TILs

	Liver cancer	Oesophag eal cancer	Gastric cancer	Colorecta l cancer	Renal cancer	Breast cancer	Thyroid cancer
Liver cancer	NS	****	****	****	****	****	****
Oesophag eal cancer	****	NS	NS	NS	NS	NS	NS
Gastric cancer	****	NS	NS	NS	NS	NS	NS
Colorectal cancer	****	NS	NS	NS	NS	NS	NS
Renal cancer	****	NS	NS	NS	NS	NS	NS
Breast cancer	****	NS	NS	NS	NS	NS	NS
Thyroid cancer	****	NS	NS	NS	NS	NS	NS

Table 3-9: Statistics box of CD160 expression on CD4 and CD8 TILs

	Liver cancer	Oesophageal cancer	Gastric cancer	Colorectal cancer	Renal cancer	Breast cancer	Thyroid cancer
Liver cancer	NS	*	*	*	*	****	*
Oesophageal cancer	*	NS	NS	NS	NS	NS	NS
Gastric cancer	*	NS	NS	NS	NS	NS	NS
Colorectal cancer	*	NS	NS	NS	NS	NS	NS
Renal cancer	*	NS	NS	NS	NS	NS	NS
Breast cancer	****	NS	NS	NS	NS	NS	NS
Thyroid cancer	*	NS	NS	NS	NS	NS	NS

In Figure 3-5, we show the results of the expression analysis of inhibitory receptors on CD4+ and CD8+ TILs from 7 different cancers. No differential expression of PD-1, BTLA and CTLA-4 on T cells was detected in the different cancer samples. Compared to the expression of Tim-3 in breast cancer, Tim-3 has higher expression on T-cells from “digestive organ” cancers such as liver cancer, esophageal cancer, gastric cancer and colorectal cancer. KLRG-1 and CD160 have higher expression on T-cells in HBV HCC individuals compared with its expression in other cancers. This up-regulation may result from the persistent HBV exposure, as the rest of the 6 types of cancer individuals are all HBV negative.

One of the most notable results is that PD-1 has a wide expression range between different cancer microenvironments and this heterogeneous expression was observed even within different individuals in the same cancer subgroup. For instance, PD-1 expression has higher expression on CD8 TILs (mean expression over 40% CD8 T cells) from liver cancer individuals, gastric individuals and renal cancer individuals. In addition, within 43 breast cancer individuals, we have observed quite a wide expression range of PD-1 on both CD4 and CD8 TILs (PD-1 expression ranges from 20% to 80% of TILs).

This raises another question that if PD-1 expression is associated with the disease stages then the advanced stage breast cancer tumor microenvironment may induce higher PD-1 expression on TILs. This was tested and is presorted later in this chapter. Furthermore, this result may also indicate that when

applying PD-1 inhibitor blockade immunotherapy to candidates, it is advisable to biopsy the tumor tissues and quantify PD-1 expression on TILs as a crucial evidence to select patients who may benefit from PD-1 blockade therapy.

Previous clinical results hypothesized PD-1's ligand, PD-L1 may serve as the biomarker to predict PD-1 inhibitor's efficacy. However, later results revealed from multiple clinical trials showed about 20% of non-PD-L1 expression advanced melanoma individuals also demonstrate clinical benefits after PD-1 inhibitor therapy[48, 210]. This makes PD-L1 as a potential biomarker more controversial. However, those facts and our data led us postulate that additional information may be obtained if clinicians quantify PD-1 expression on TILs from biopsy tissues prior to the blockade treatment in cancer patients.

Tim-3 has a preferential expression according to the type of cancers. Our data in Figure 3-5 suggests that Tim-3 preferentially expressed in digestive organ cancers such as liver cancer, gastric cancer, esophageal cancer and colorectal cancer. Previous study of Tim-3 in HCV chronically infected individuals has indicated that the co-expression of PD-1 and Tim-3 on intrahepatic lymphocytes is associated with poor outcome and more dysfunctional T cells[211]. In our Tim-3 expression analysis on TILs, we have found the good prognosis cancers such as breast cancer and thyroid cancer have relatively low Tim-3 expression on both CD4 and CD8 TILs compared with poor prognosis cancer types such as liver cancer, esophageal cancer, colorectal cancer and gastric cancer. However, given the facts that Tim-3 also expressed on T cell from normal colorectal tissues, this preferential expression may not be a specific up-regulation as digestive organs need to limit autoimmunity and immunopathology as they have direct

contact with numerous foreign antigens[107]. Hence Tim-3 expression on T cells of those digestive organ cancers may be a physiological consequence to avoid over-activation of resident T cells. To further answer this question, appropriate benign tissues are essential controls.

CTLA-4 has been thought as promising target in multiple cancers. But in our study, we have observed that it has relatively low expression (mean expression is less than 20% of T cells) on TILs in 7 types of cancers. This may indirectly explains why when ipilimumab extends its application to other cancers, its clinical benefits are limited but its immune-related side effects are lethal[78]. As mentioned previously, its expression was predominately on Tregs on which we could not distinguish it from conventional CD4 T cells[42]. Hence, our study could not provide information about CTLA-4 expression on tumor infiltrating Tregs from different tumor microenvironments. But this would be an opportunity for future investigation.

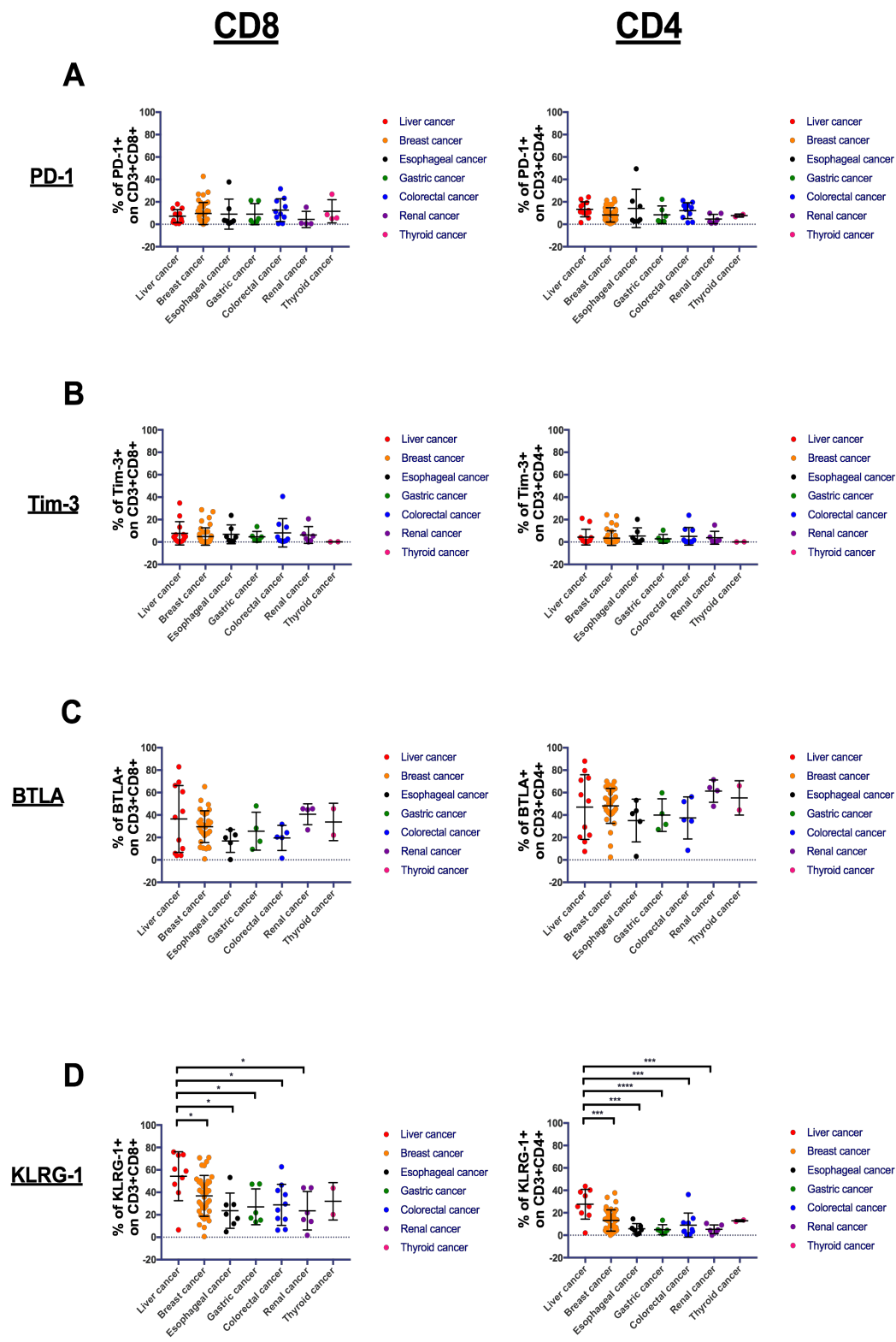
Interestingly, KLRG-1 and CD160 have higher expression on TILs in liver cancer when compared with other types of cancer. Despite different tumor microenvironments may alter the phenotypes of tumor infiltrating T cells, the significant higher expression of KLRG-1 and CD160 in liver cancer may result from continuous exposure of HBV virus as all the liver cancer individuals are HBV chronically infected individuals. Dual factors, chronic virus infection and the establishment of cancer may contribute to a more exhausted T-cell phenotype with up-regulation of KLRG-1 and CD160. Given the facts that KLRG-1 is also a T-cell differentiation marker, without functional assays, we could not

confirm whether its upregulation is mainly resulted from chronic HBV infection. To further test this hypothesis, non-virus infected primary liver cancer individuals should be recruited. But our data at least shed a light on KLRG-1, as the potential blockade target in treating chronically HBV infected HCC individuals.

3.2.3 Less variable sole inhibitory receptor expression on circulating T cells from different cancer individuals

Having compared the expression of inhibitory receptors on tumor infiltrating T-cells in different tumor microenvironments, we further analyzed the paired fresh peripheral blood from patients in the same cohort. In such an approach, it enables us to investigate whether the differential expression pattern of sole inhibitory receptor on TILs from different cancers would reflect in the T-cells in circulating blood. In addition, the general expression of sole inhibitory receptor on T cells in the peripheral blood could also indicate the potential side effects when applying antibody blockade therapy.

Figure 3-6: Expression of sole inhibitory receptor on peripheral blood from different cancers



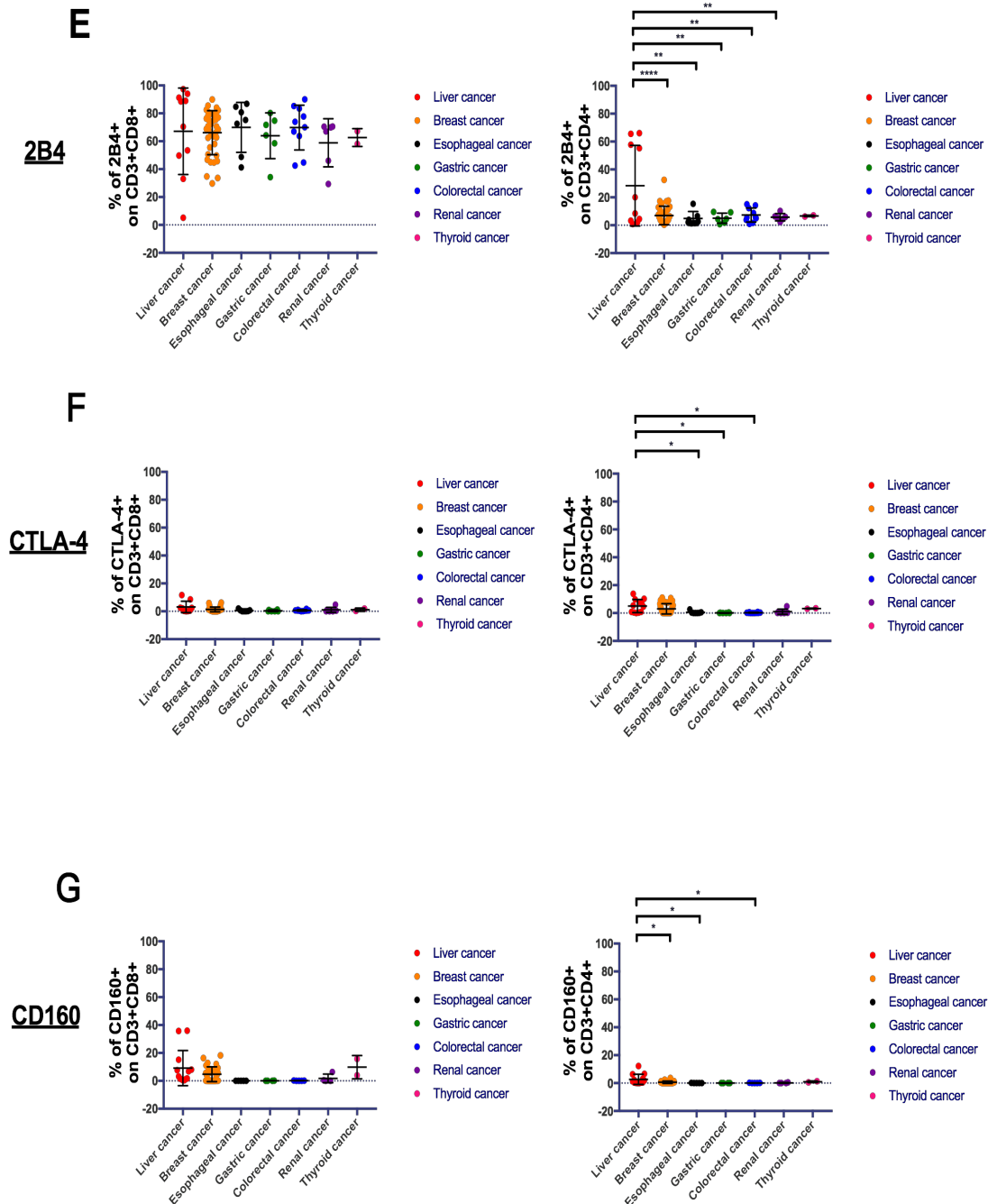


Figure 3-6: *ex vivo* expression analysis of PD-1, TIM-3, BTLA, KLRG-1, 2B4, CTLA-4 and CD160 on CD8+ (left graph) and CD4+ T-cells (right graph) TILs from HBV associated liver cancer, breast cancer, esophageal cancer, gastric cancer, colorectal cancer, renal cancer and thyroid cancer patients. The analysis includes 16 HBV associated HCC patients, 43 breast cancer patients, 6 esophageal cancer patients, 7 gastric cancer patients, 8 colorectal cancer patients, 5 renal cancer patients and 3 thyroid cancer patients (* $P < 0.0332$, ** $P < 0.0021$, *** $P < 0.0003$, **** $P < 0.0001$ ANOVA multiple t test). **A)** PD-1, **B)** TIM-3, **C)** BTLA, **D)** KLRG-1, **E)** 2B4, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ TILs in (%).

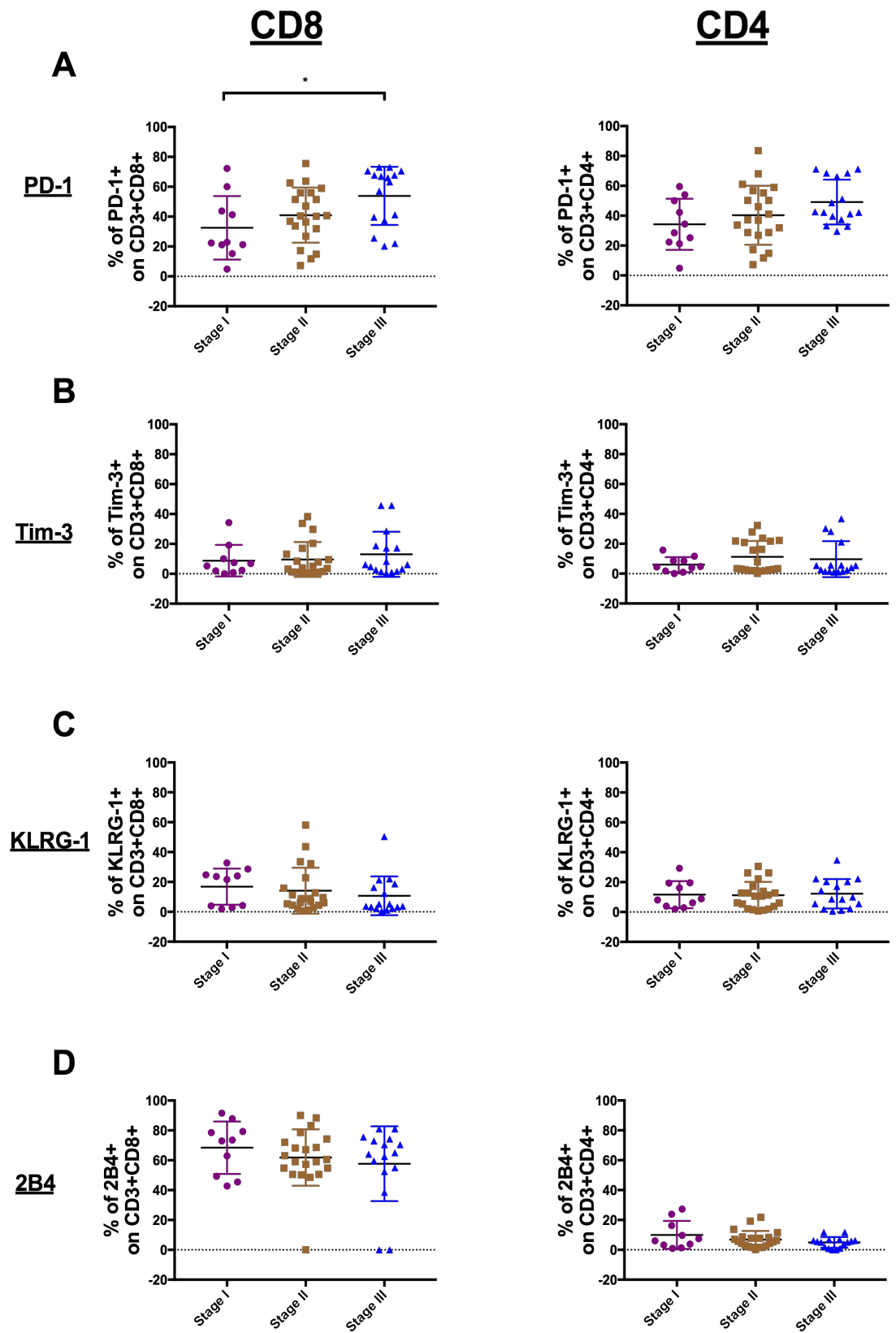
In figure 3-6, generally, lower differential expression levels of sole inhibitory receptors were found in peripheral blood from 7 different cancer individuals when compared with cancer tissues from patients in the same cohort. No differential expression of PD-1, Tim-3 and BTLA on T cells was observed within 7 different cancers. However, the expression pattern of KLRG-1 in circulating T-cells among 7 cancers is in line with that in the tumor microenvironments. Indeed, the expression of KLRG-1 on T cells from PBMCs in HBV HCC individuals is higher than that in other cancers.

In conclusion, by comparing the expression of sole inhibitory receptors on T cells from both tumor tissues and PBMCs, we have noticed that PD-1, Tim3 and KLRG-1 have heterogeneous expression on T cells in different cancers. Based on the general expression of inhibitory receptors on T cells from tumor tissues, PD-1 has the potential to be the “universal” target when considering a sole inhibitory pathway blockade immunotherapy as 4 out of 7 solid tumors have on average over 40% expression of PD-1 on CD8⁺ TILs. In addition, Tim-3 may be treated as a supplementary target in “digestive organ” cancers owing to the higher expression on CD8⁺ TILs in liver cancer, esophageal cancer, gastric cancer, and colorectal cancer. In the development of sole inhibitory receptor blockade therapy, CD160, CTLA-4 and BTLA may be less promising as the global expression levels on TILs are quite low when comparing with the other inhibitory receptors we chose, although anti-CTLA-4 mAb has been approved as the first inhibitory pathway blockade medication by FDA[102, 212].

3.2.4 Stage based *ex vivo* characterization of sole expression of inhibitory receptors on T cells from breast cancer individuals

With the useful number of breast cancer samples obtained from Xinjiang Cancer Hospital in China, we further analyzed the expression of sole inhibitory receptor of PBMCs and TILs among different stages of breast cancer individuals. In total, we have collected 43 fresh surgical breast cancer tissue samples as well as the paired peripheral blood, of which 10 individuals are TNM stage I, 19 individuals are TNM stage II and 14 individuals are stage III (details are shown in Table 3-3). TNM classification has been widely applied as the survival model to integrally evaluate tumors burden, involvement of adjacent lymph nodes and distant metastasis[213]. This analysis is to test that if the expression of any sole inhibitory receptor on T cells from PBMCs or TILs is associated with the tumor progression, which may reveal useful information for the application of sole inhibitory pathway blockade in tumor stage based manner.

Figure 3-7: Expression of sole inhibitory receptor on tumor infiltrating T cells from breast cancer individuals in differential TNM stages



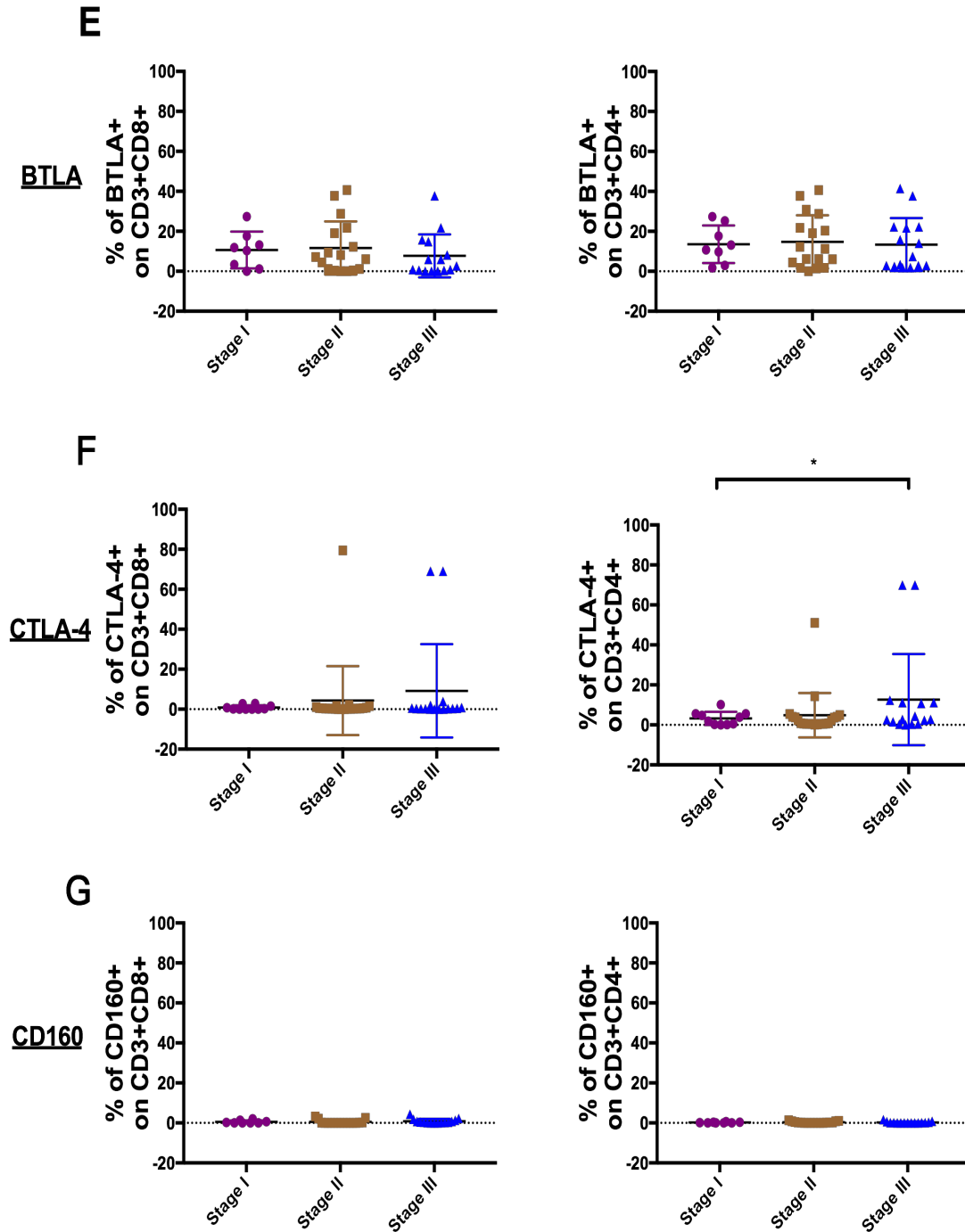
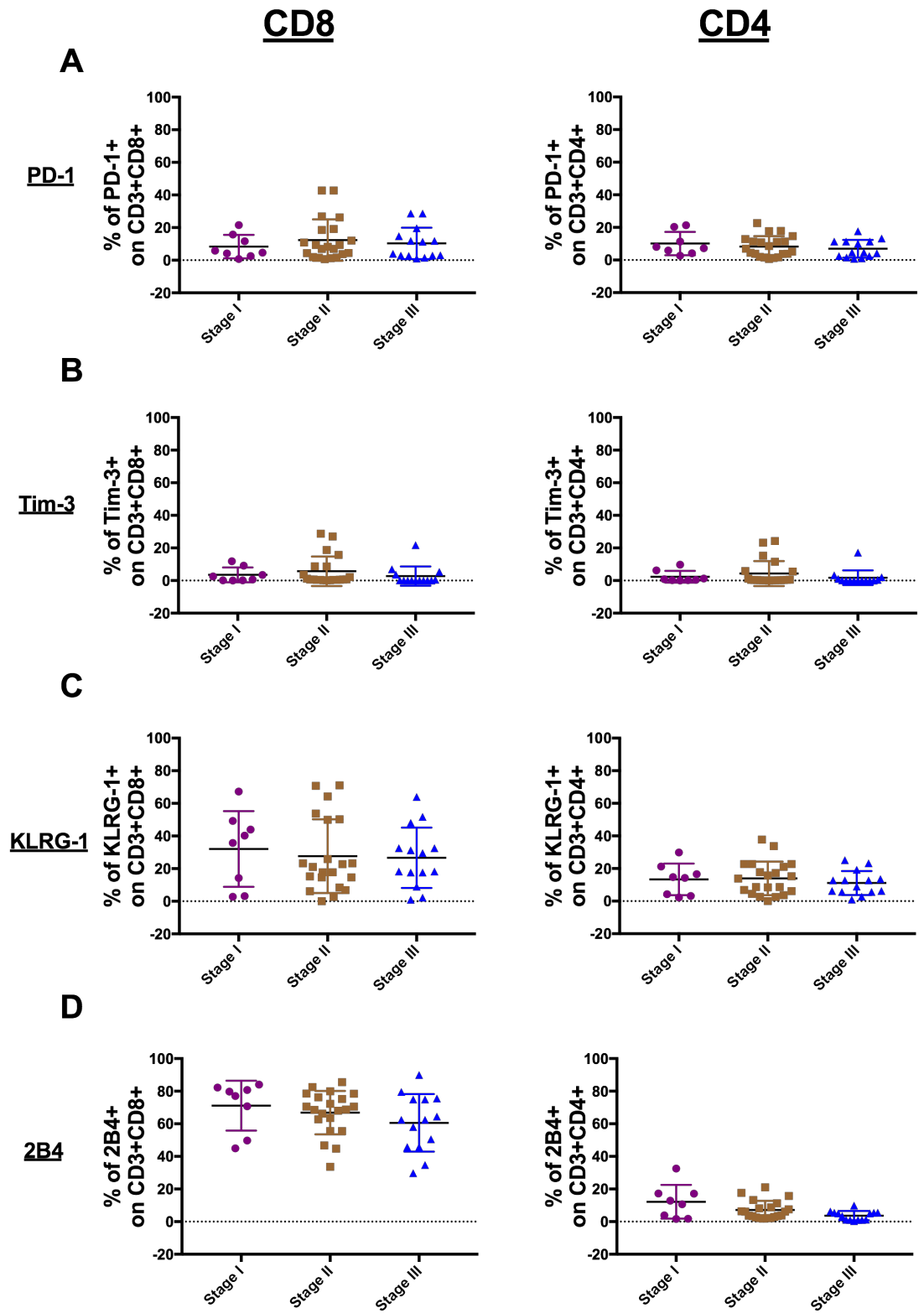


Figure 3-7: *ex vivo* expression analysis of PD-1, TIM-3, KLRG-1, 2B4, BTLA, CTLA-4 and CD160 on CD8+ (left graph) and CD4+ T-cells (right graph) TILs from 34 primary breast cancer patients, of which 10 individuals are TNM stage I, 19 individuals are TNM stage II and 14 individuals are stage III (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitneyt-test). **A)** PD-1, **B)** TIM-3, **C)** KLRG-1, **D)** 2B4, **E)** BTLA, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ TILs in (%).

Figure 3-8: Expression of sole inhibitory receptor on peripheral T cells from breast cancer individuals in differential TNM stages



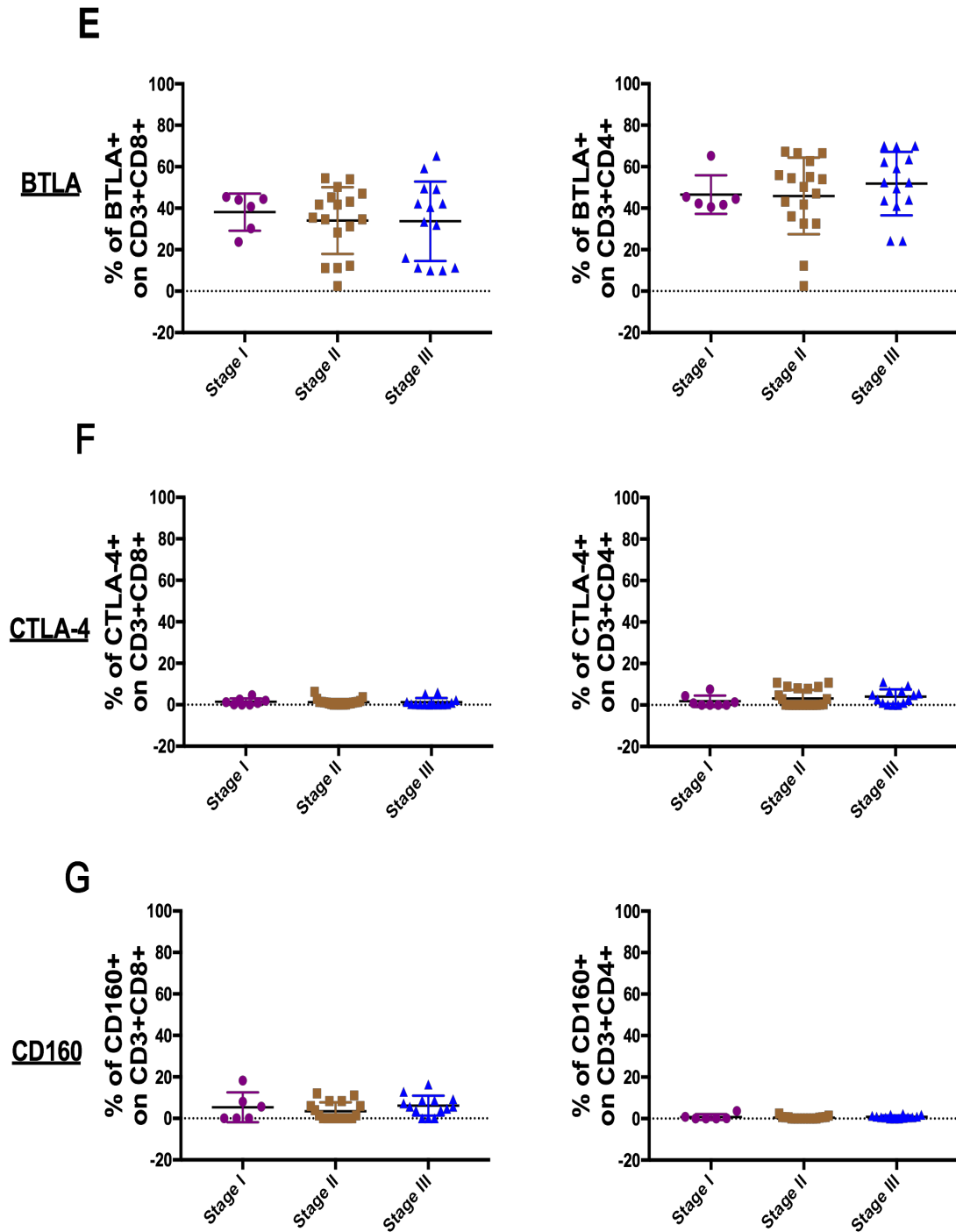


Figure 3-8: *ex vivo* expression analysis of PD-1, TIM-3, KLRG-1, 2B4, BTLA, CTLA-4 and CD160 on CD8+ (left graph) and CD4+ T-cells (right graph) PBMCs from 34 primary breast cancer patients, of which 10 individuals are TNM stage I, 19 individuals are TNM stage II and 14 individuals are stage III (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney t-test). **A)** PD-1, **B)** TIM-3, **C)** KLRG-1, **D)** 2B4, **E)** BTLA, **F)** CTLA-4, **G)** CD160 expression on CD4+ and CD8+ TILs in (%).

With 43 surgical samples from breast cancer individuals, we have got the chance to examine the expression of inhibitory receptors on T-cells and the association with tumor progression. Patients are stratified by the TNM staging system. To our surprise, the majority of inhibitory receptors on TILs or PBMCs from different TNM stages breast cancer individuals have no differential expression from one another. Only PD-1 on CD8+ TILs has higher expression in stage III breast cancer individuals compared with that in stage I breast cancer individuals. Although CTLA-4 on CD4+ TILs has slightly higher expression in stage III breast cancer individuals compared with that in stage I breast cancer individuals, the global expression level of CTLA-4 on TILs is rather low (below 20% on average).

It should be noted that we have not conducted functional experiments on TILs from breast cancer individuals in different stages. This means that TILs from different stages breast cancer individuals may exhibit similar phenotypes characterized by the expression of currently known inhibitory receptors. But they may demonstrate differential anti-tumor function. In other words, advanced stage breast cancer may harvest more exhausted T cells with poor cytokines profiling and higher proportion of functionally non-reversible T cells, whereas early stage and intermediate stage breast cancer may consist of less exhausted T cells with higher proportion of functionally reversible T cells. In addition, multiple inhibitory receptors may be used as the activation markers when T-cells differentiated into effector phenotypes[214]. To fully characterize T-cell exhaustion in breast cancer from different stages, functional studies of

TILs should be incorporated along with its inhibitory receptors expression on TILs.

In conclusion, this set of data indicates that sole inhibitory receptors have limited implications for the design of future blockade immunotherapy owing to its heterogeneous expression on bulk T-cells from tumors. To identify the effective targets within multiple available inhibitory receptors, further characterization in combinatorial expression of inhibitory receptors is necessary.

3.2.5 Identification of combinatorial expression of inhibitory receptors on CD8+ tumor infiltrating T cells from different cancer individuals

Blocking inhibitory receptor signals of tumor infiltrating T-cells in tumor microenvironments has become one of the most promising approaches to resuscitate anti-tumor immune responses against immunosuppressiveness[215, 216]. Positive results of PD-1/PD-L1 blockade immunotherapy for multiple cancers have been achieved. However, still, a fair number of patients are non-responders to PD-1/PD-L1 blockade treatment, which indicates the potential to discover alternative inhibitory pathways[171]. In addition, sole inhibitory pathway blockade, such as PD-1/PD-L1 and CTLA-4/galectin9 have been intensively investigated, while the co-blocking regimen has yet to be characterized. Clinically, sole inhibitory receptor expression and its ligand expression in a tumor microenvironment is the vital indication for blockade immunotherapy and patients' prognosis. A further question after the characterization of sole inhibitory receptor expression on T-cells is to identify

the dominant tumor infiltrating T-cell phenotype by multiple inhibitory receptors in different cancer microenvironments, which may help the development of the rationale for combinatorial inhibitory pathway blockade. Hence, based on the results of the expression of sole inhibitory receptors on CD8⁺ TILs, and the report of the newly identified inhibitory receptor, we can apply combinatorial expression analysis of PD-1, 2B4, KLRG-1, TIGIT and CTLA-4 using SPICE (Simplified Presentation of Incredibly Complex Evaluations) to characterize tumor infiltrating T-cell phenotype and to investigate the potential combinatorial blockade immunotherapy regimen.

Figure 3-9: Combinatorial expression analysis of co-inhibitory receptors pattern on CD8 tumor infiltrating T cells in HBV HCC individuals

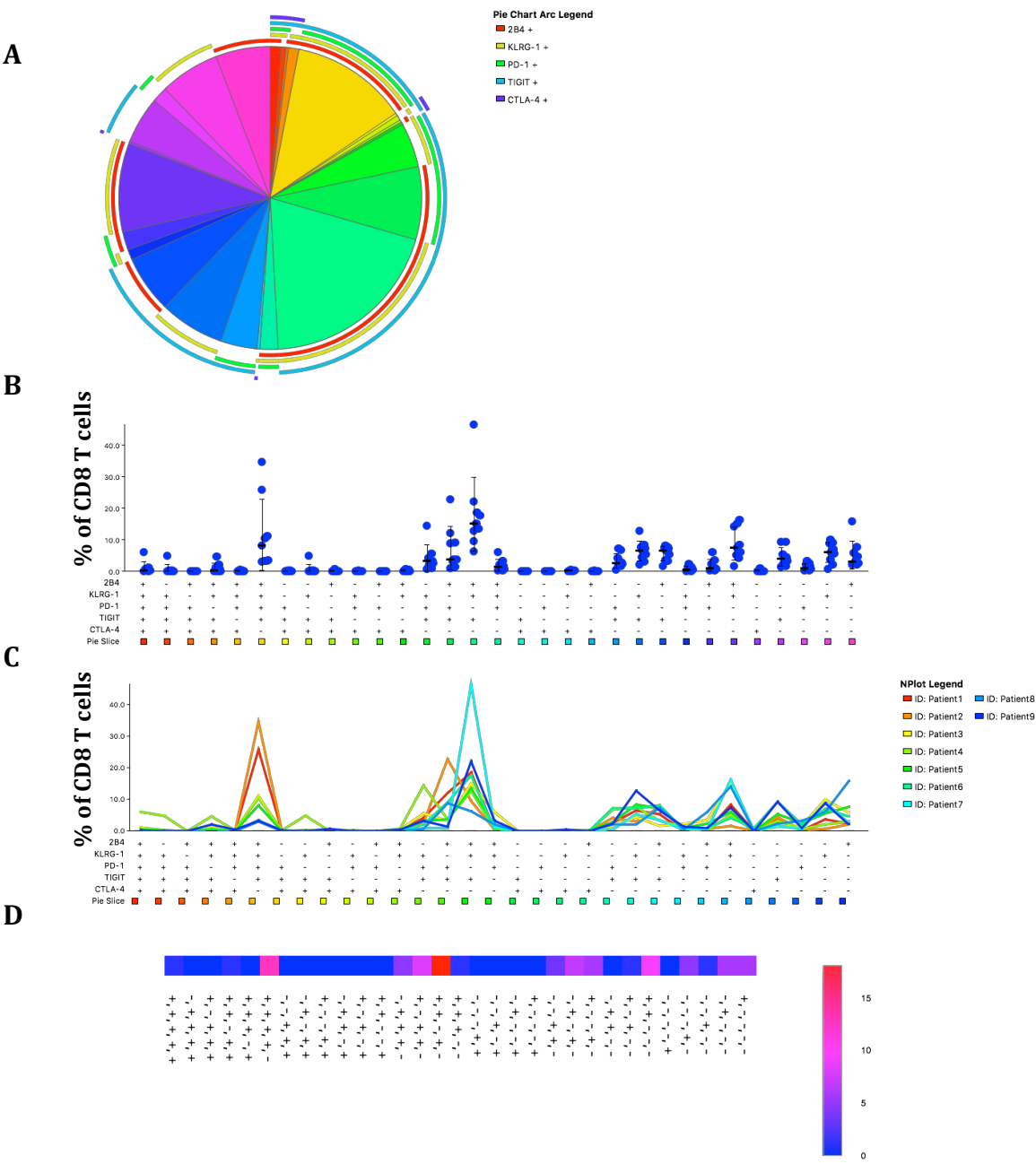


Figure 3-9: (a) Pie chart depicts the percentage of different subsets in CD8 TILs characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4). The outer arcs illustrated the sole inhibitory receptor expression in different subsets. **(b)** Boolean gating was applied to depict the dot plot that demonstrated the frequency of 31 subsets characterized by 5 inhibitory receptors from CD8 TILs. **(c)** Single color line represents frequency of different subsets of the individual patient and is consistent to the above dot plot. **(d)** Heat map was drawn to identify the dominated and sub-dominated subsets in CD8 TILs in 9 HBV HCC individuals

Figure 3-10: Combinatorial expression analysis of co-inhibitory receptors pattern on CD8 tumor infiltrating T cells in breast cancer individuals

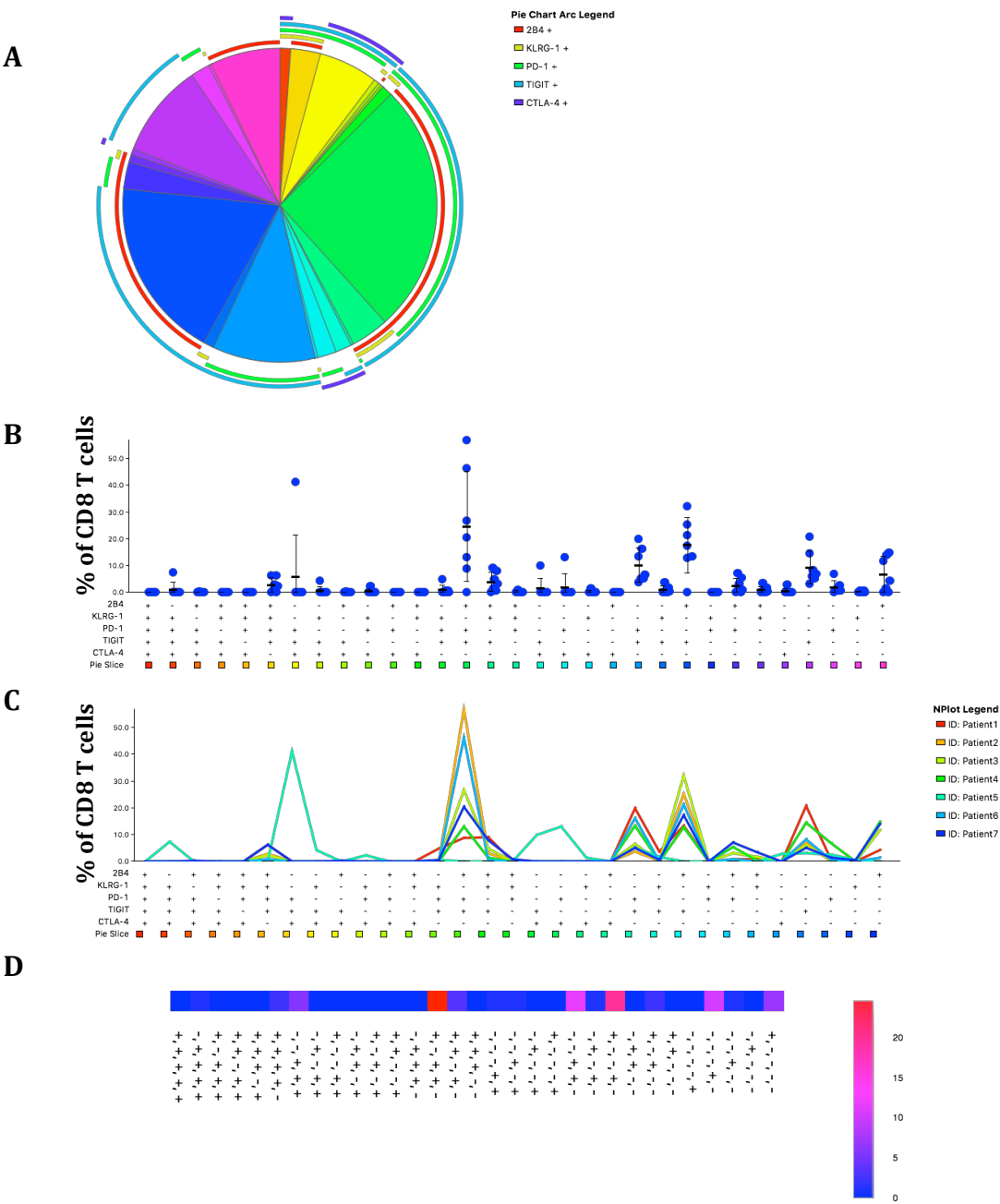


Figure 3-10: (a) Pie chart depicts the percentage of different subsets in CD8 TILs characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4). The outer arcs illustrated the sole inhibitory receptor expression in different subsets. **(b)** Boolean gating was applied to depict the dot plot that demonstrated the frequency of 31 subsets characterized by 5 inhibitory receptors from CD8 TILs. **(c)** Single color line represents frequency of different subsets of the individual patient and is consistent to the above dot plot. **(d)** Heat map was drawn to identify the dominated and sub-dominated subsets in CD8 TILs in 7 breast cancer individuals.

Figure 3-11: Combinatorial expression analysis of co-inhibitory receptors pattern on CD8 tumor infiltrating T cells in colorectal cancer individuals

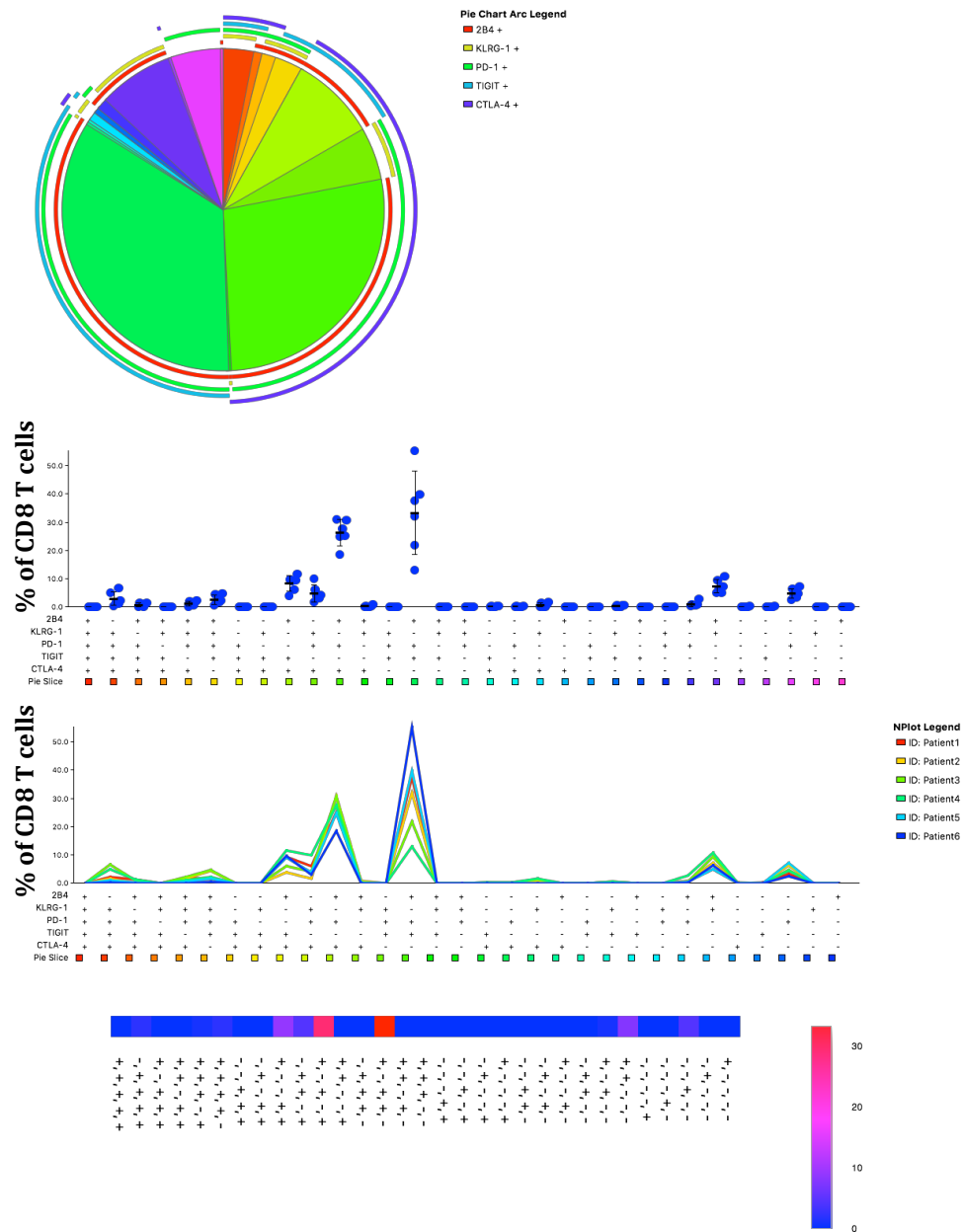


Figure 3-11: (a) Pie chart depicts the percentage of different subsets in CD8 TILs characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4). The outer arcs illustrated the sole inhibitory receptor expression in different subsets. **(b)** Boolean gating was applied to depict the dot plot that demonstrated the frequency of 31 subsets characterized by 5 inhibitory receptors from CD8 TILs. **(c)** Single color line represents frequency of different subsets of the individual patient and is consistent to the above dot plot. **(d)** Heat map was drawn to identify the dominated and sub-dominated subsets in CD8 TILs in 6 colorectal cancer individuals.

Figure 3-12: (a) Pie chart depicts the percentage of different subsets in CD8 TILs characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4). The outer arcs illustrated the sole inhibitory receptor expression in different subsets. **(b)** Boolean gating was applied to depict the dot plot that demonstrated the frequency of 31 subsets characterized by 5 inhibitory receptors from CD8 TILs. **(c)** Single color line represents frequency of different subsets of the individual patient and is consistent to the above dot plot. **(d)** Heat map was drawn to identify the dominated and sub-dominated subsets in CD8 TILs in 4 gastric cancer individuals.



Figure 3-13: Combinatorial expression analysis of co-inhibitory receptors pattern on CD8 tumor infiltrating T cells in esophageal cancer individuals

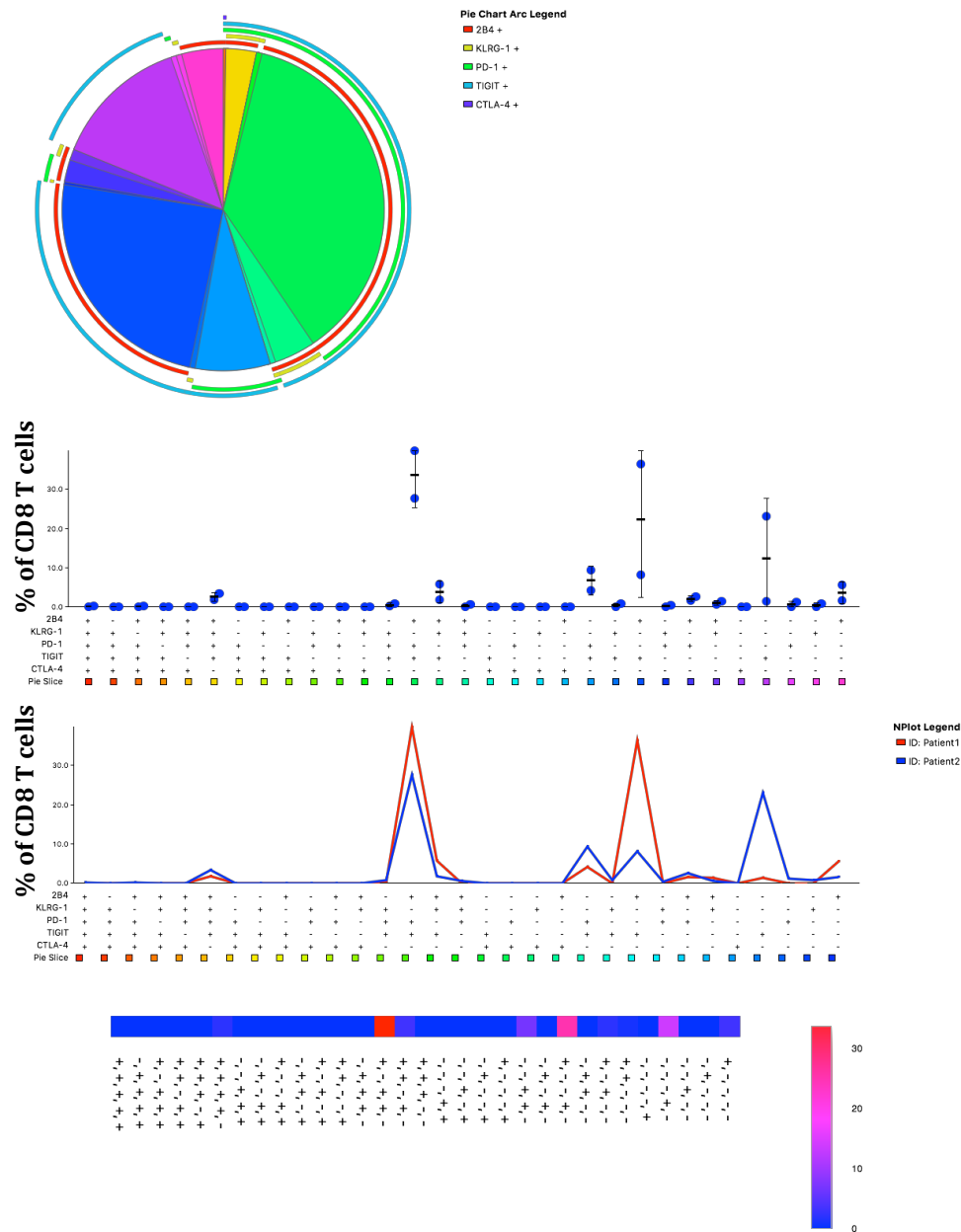


Figure 3-13: (a) Pie chart depicts the percentage of different subsets in CD8 TILs characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4). The outer arcs illustrated the sole inhibitory receptor expression in different subsets. **(b)** Boolean gating was applied to depict the dot plot that demonstrated the frequency of 31 subsets characterized by 5 inhibitory receptors from CD8 TILs. **(c)** Single color line represents frequency of different subsets of the individual patient and is consistent to the above dot plot. **(d)** Heat map was drawn to identify the dominated and sub-dominated subsets in CD8 TILs in 2 esophageal cancer individuals.

Figure 3-14: Combinatorial expression analysis of co-inhibitory receptors pattern on CD8 tumor infiltrating T cells in lung cancer individuals

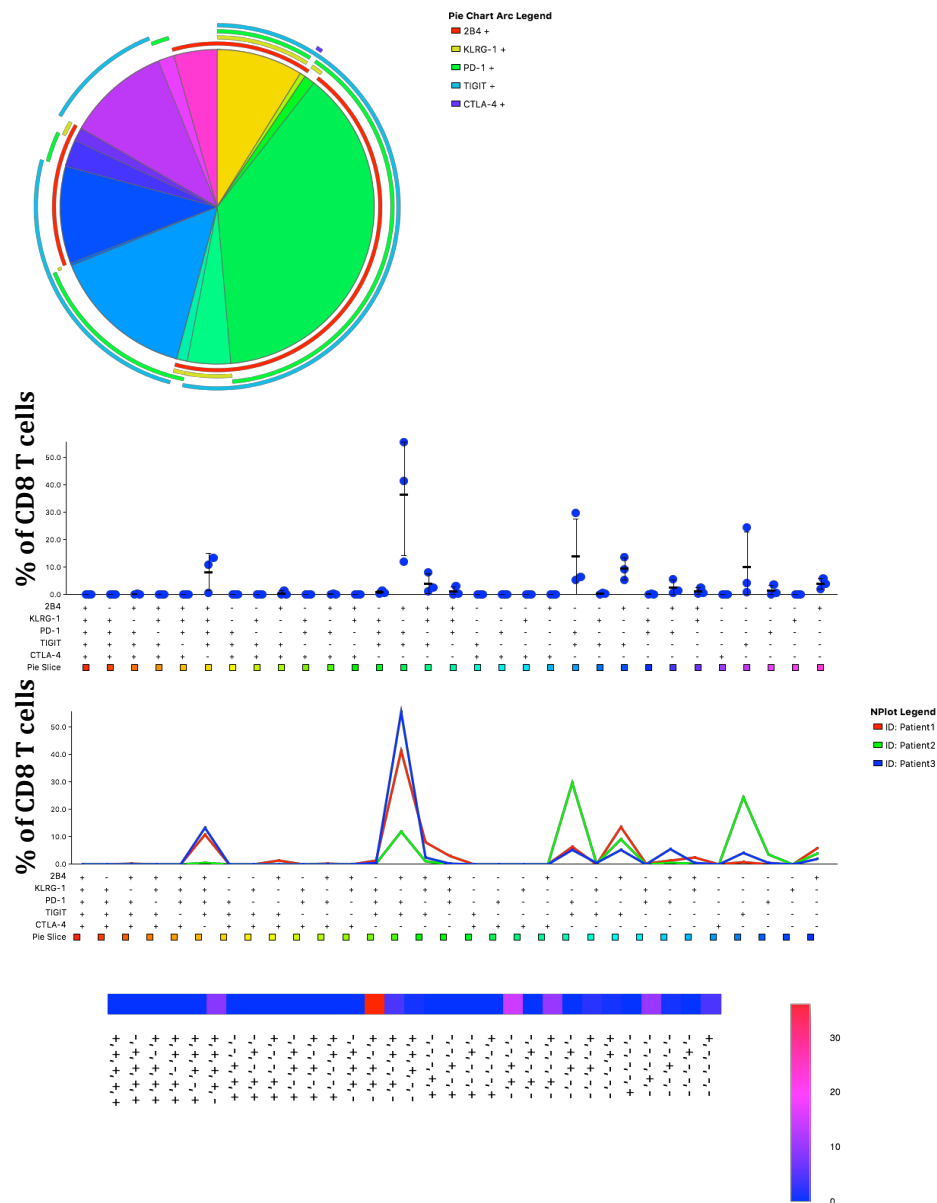


Figure 3-14: (a) Pie chart depicts the percentage of different subsets in CD8 TILs characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4). The outer arcs illustrated the sole inhibitory receptor expression in different subsets. **(b)** Boolean gating was applied to depict the dot plot that demonstrated the frequency of 31 subsets characterized by 5 inhibitory receptors from CD8 TILs. **(c)** Single color line represents frequency of different subsets of the individual patient and is consistent to the above dot plot. **(d)** Heat map was drawn to identify the dominated and sub-dominated subsets in CD8 TILs in 3 lung cancer individuals.

Figure 3-15: Combinatorial expression analysis of co-inhibitory receptors pattern on CD8 tumor infiltrating T cells in renal cancer individuals

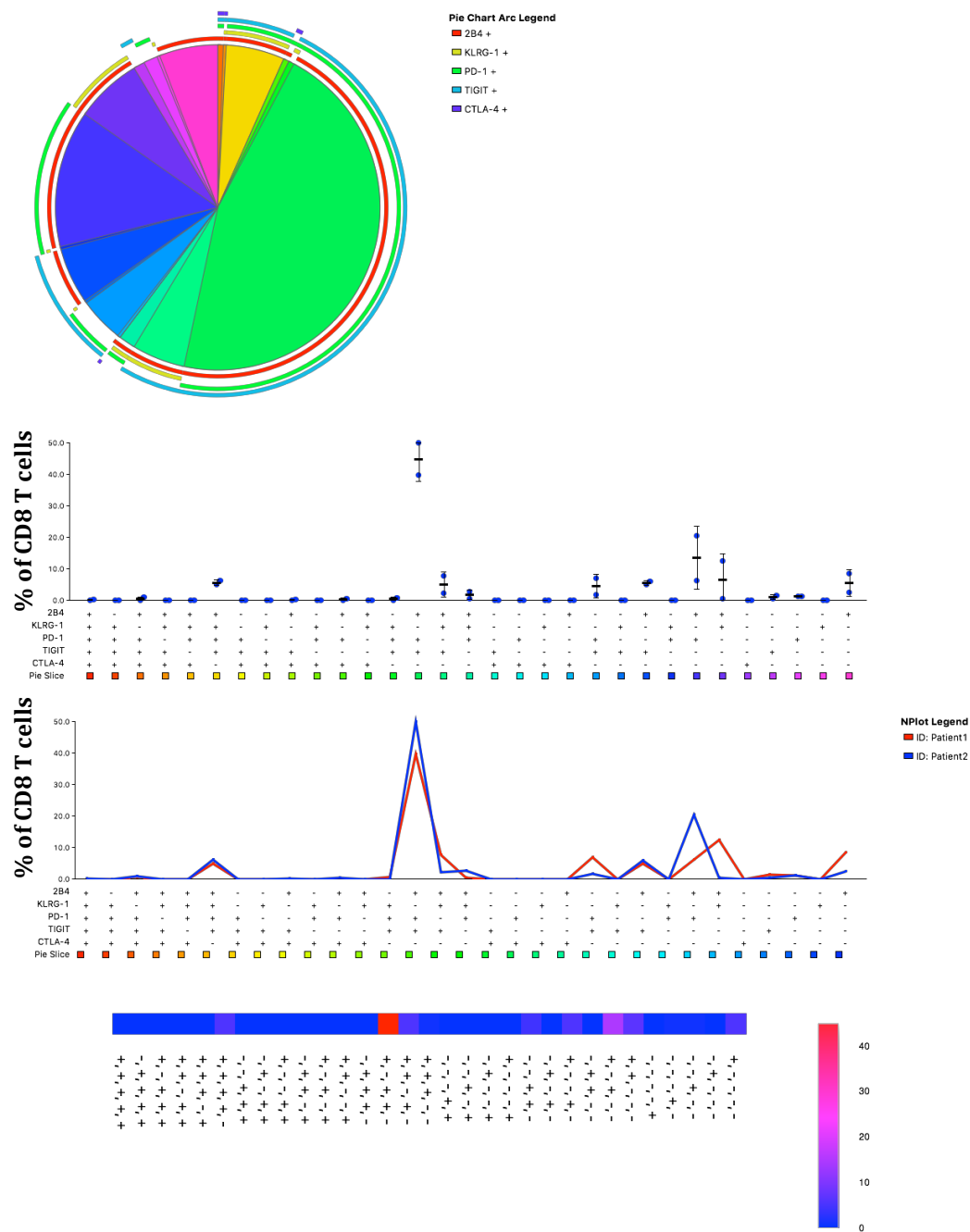


Figure 3-15: (a) Pie chart depicts the percentage of different subsets in CD8 TILs characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4). The outer arcs illustrated the sole inhibitory receptor expression in different subsets. (b) Boolean gating was applied to depict the dot plot that demonstrated the frequency of 31 subsets characterized by 5 inhibitory receptors from CD8 TILs. (c) Single color line represents frequency of different subsets of the individual patient and is consistent to the above dot plot. (d) Heat map was drawn to identify the dominated and sub-dominated subsets in CD8 TILs in 2 renal cancer individuals.

In the clinical trials of CTLA-4 and PD-1 inhibitors, researchers and clinician have observed a certain subgroup of patients that could not benefit from blockade immunotherapy[73]. To extend the application of inhibitory pathway blockades, on the one hand, more additional inhibitory receptors are promising targets; on the other hand, combinatorial blockades of multiple inhibitory pathways simultaneously may allow more patients achieve benefits. In melanoma blockade therapy, combined application of PD-1 inhibitor and CTLA-4 inhibitor improved its efficacy and sustain its anti-tumor responses with prolonged overall survival[217-219]. Therefore, to characterize which combinatorial regimen would be optimal and to tailor personal precision blockade regimen, we dissected CD8 TILs by PD-1, CTLA-4, KLRG-1, TIGIT and 2B4 expression.

SPICE (Simplified Presentation of Incredibly Complex Evaluations) was designed to reveal the complexity of multidimensional data[220]. In our studies, SPICE analysis was applied to subdivide CD8⁺ TILs into 31 subsets by differential combination of 5 inhibitory receptors. CD8⁺ TILs from HBV associated HCC (Figure 3-9) were dominated by 2B4+KLRG-1+TIGIT+PD-1-CTLA-4- subset and were sub-dominated by 2B4+KLRG-1+PD-1-TIGIT-CTLA-4- and 2B4+KLRG-1+PD-1+TIGIT+CTLA-4- subsets. Interestingly, breast cancer, colorectal cancer, gastric cancer, esophageal cancer, lung cancer and renal cancer share the same dominant subset, 2B4+PD-1+TIGIT+KLRG-1-CTLA-4- but different sub-dominant subsets (figure 3-10 to 3-15), although the sample size of 6 cancers were limited and needs to be extended, the combinatorial expression of

inhibitory receptors on CD8⁺ TILs was observed. This data implies that in chronically HBV infected HCC individuals, 2B4, KLRG-1 and TIGIT may be targeted in the regimen of multiple inhibitory receptors blockade therapy but not PD-1. Notably, it was found that in HBV HCC CD8⁺ TILs, the PD-1 negative subsets are predominately KLRG-1 positive. This may shed a light on sustaining anti-tumor T-cell responses to PD-1 non-responders by combining KLRG-1, TIGIT and 2B4 blockades, although thorough functional characterization of those blockades should first be conducted. As in breast cancer individuals, esophageal cancer individuals, gastric cancer individuals, lung cancer individuals and renal cancer individuals share the same dominated subset in CD8⁺ TILs, which is 2B4⁺PD-1⁺TIGIT⁺KLRG-1⁺CTLA-4⁻ subset. 2B4, PD-1 and TIGIT may involve in the combinatorial blockade regimen in treating those cancers.

To our knowledge, this is the first system proposed to analyze combinatorial expression pattern on TILs in different cancers aiming to provide rationale in future combinatorial inhibitory pathways blockade. With more newly identified inhibitory receptors and upgraded flow cytometry machine, it enables us to not only identify the dominated phenotype as a reference for combinatorial blockade, but also conduct functional analysis on “targeted” subsets by sorting the dominated phenotype TILs in different cancers and in one cancer with different disease stages.

3.2.5 Implementing “immunoscores” of CD8+ TILs to dissect T cells from different cancer environments

Conventionally, TNM classification has been thought as the standard staging system for solid tumor diagnosis. It consists of 3 elements, tumor burden (T), lymph node involvement (N) and metastasis (M)[213]. With numerous epidemiologic studies in multiple cancers, it has developed to predict patients' prognosis and evaluate tumors' pathologic features. Previously, good evidence has been demonstrated that lymphocytic infiltration is a positive indication of patients' outcome[221, 222]. Recently, in combining with the noteworthy benefits inhibitory pathway blockade immunotherapies achieved, it is plausible to incorporate the infiltrating lymphocytes in tumor microenvironments into the diagnosis and survival model regardless of the heterogeneity of cancer from patient to patient. Hence, we proposed the notion of “immunoscores” of CD8+ tumor infiltrating T lymphocytes in order to individually assess T-cell exhaustion corresponding to the rationale of precision medicine and compare the combinatorial inhibitory receptors expression pattern of T-cell exhaustion from different cancers, which in turn may yield useful information for the development of potent anti-tumor immune responses. In addition, with the involvement of more subjects' involvement and further functional characterization, it may enable us to target the dominated and sub-dominated CD8+ TILs phenotypes from different tumor microenvironments and design relevant combinatorial blockade immunotherapy.

In this primary scoring analysis of CD8+ tumor infiltrating lymphocytes from 7 different cancers, we simply define the scores of different subsets based on the

number of positive inhibitory receptors it expresses although according to the work of others, in the development of T-cell exhaustion, inhibitory receptors may express in a certain sequential order[200, 223]. In addition, the expression of different inhibitory receptors may contribute to T-cell exhaustion in different grades. Furthermore, it has been suggested that several inhibitory receptors have preferential expression in normal tissues[107]. For instance, TIGIT is preferentially expressed in normal lung tissues, whereas, Tim-3 is selectively expressed in brain and gut tissues[107]. Hence, given those facts and the specificity of inhibitory receptors towards T-cell exhaustion, we understand and would correct this scoring system with more functional experiments in the future.

Figure 3-16: Scoring method based on the combinatorial expression inhibitory receptors on CD8+ tumor infiltrating T cells

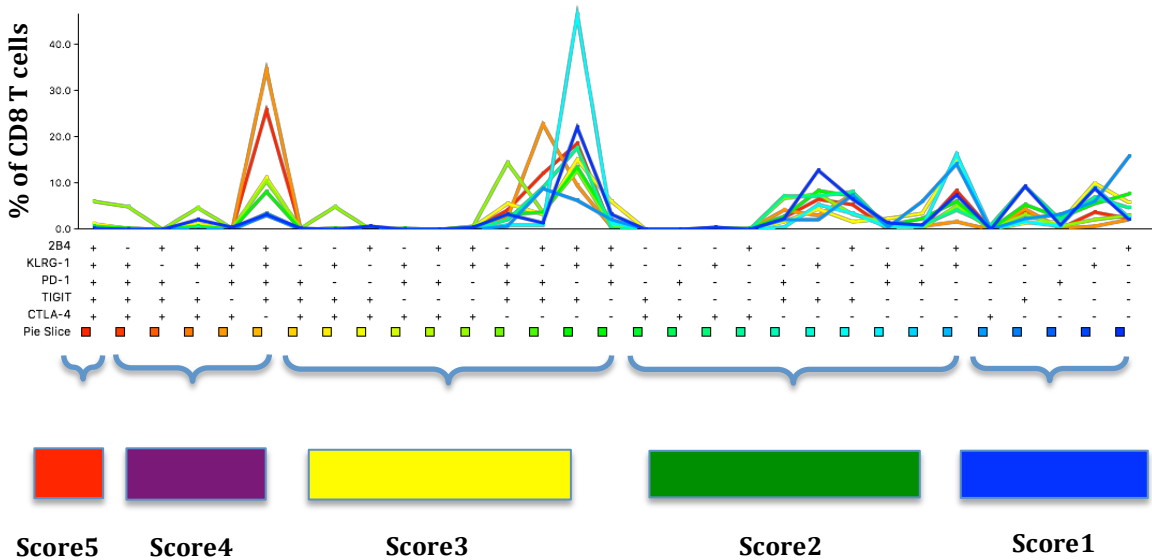


Figure 3-16: CD8 TILs are characterized by 5 inhibitory receptors (2B4, KLRG-1, PD-1, TIGIT and CTLA-4), and are divided into 31 subsets. 5 out of 5 inhibitory receptors are positive defined as 5 scores (1 subset). 4 out of 5 inhibitory receptors are positive defined as 4 scores (5 subsets). 3 out of 5 inhibitory receptors are positive defined as 3 scores (10 subsets). 2 out of 5 inhibitory receptors are positive defined as 2 scores (10 subsets). 1 out of 5 inhibitory receptors is positive defined as 1 score (5 subsets).

Figure 3-17: Immunoscores of CD8+ tumor infiltrating T cells from different cancers

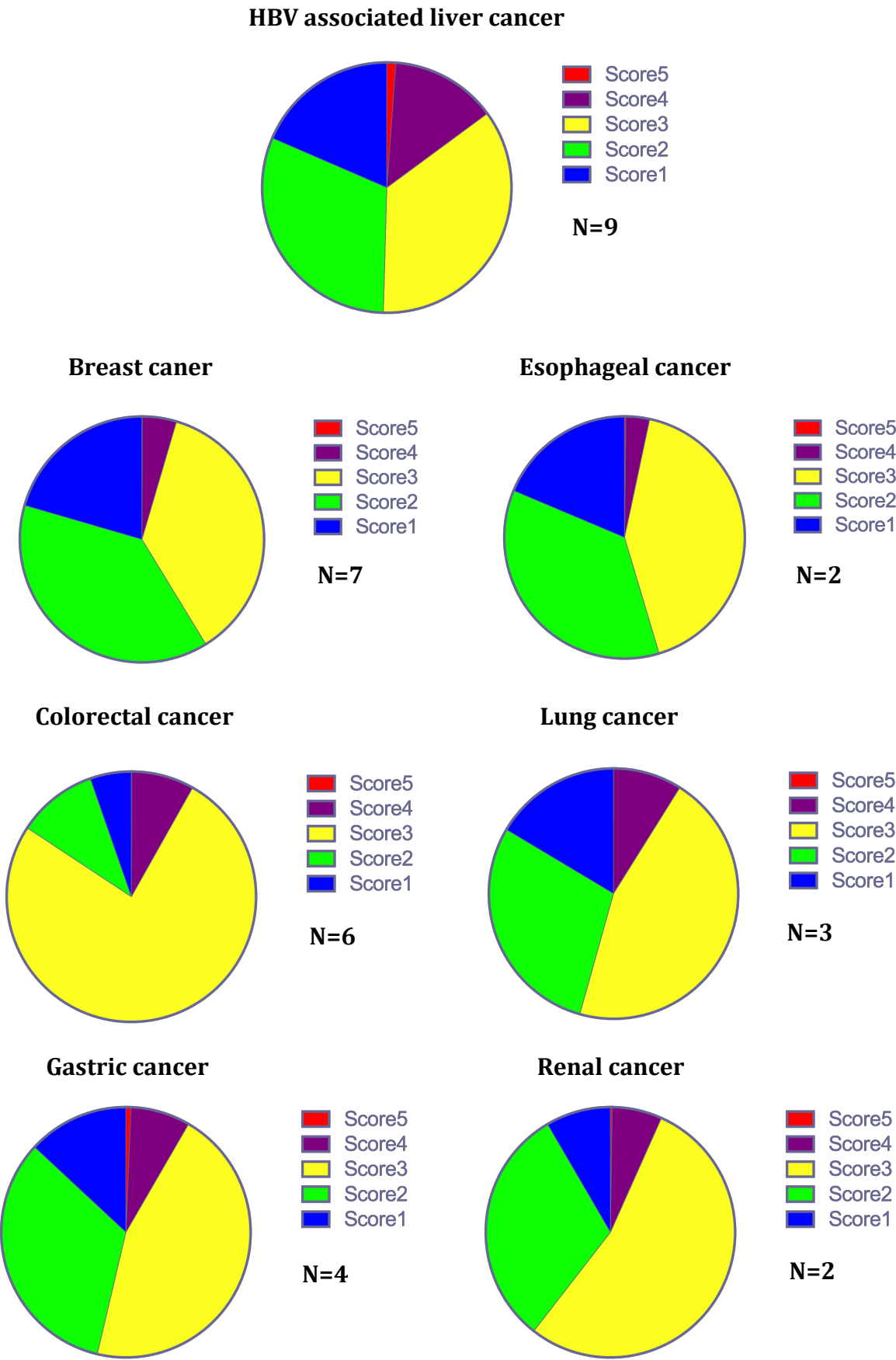


Figure 3-17: Pie charts depict the percentage of subsets in different scores. Average of the frequency in each subset was calculated from different patients in same cancer cohort. Top pie chart: immunoscores of HBV associated HCC, 3 left row pie charts: immunoscores of breast cancer, colorectal cancer and gastric cancer. 3 right row pie charts: immunoscores of esophageal cancer, lung cancer and renal cancer.

Although limited samples are available in each type of cancer, we still could observe the differential components of CD8⁺ TILs in the context of immunoscores from different tumor microenvironments. Generally, less expression of inhibitory receptors may correlate with less exhausted phenotype, although in vitro functional studies are needed. Score 1 to Score 3 may have more chance to re-invigorate its anti-tumor potency, whereas, Score 4 and Score 5 may indicate the non-reversal phenotype of dysfunctional T cells in the tumor microenvironment. It is noteworthy that Score 3 percentage in colorectal cancer is over 60%, while it only consists about 35% to 50% in other cancers. Again, this scoring system is a primarily thought to quantify the T-cell exhaustion from different cancers. Further functional studies and characterization of partially reversal and non-reversal CD8⁺ TILs should be introduced to refine this scoring system.

It has been proposed by other studies that the inhibitory receptors expression contribute to T-cell exhaustion in a “tiered” manner. Within this pyramid, the highest tier is CTLA-4 as it elicits severe autoimmune responses when blocking its pathway; intermediate “tier” is Tim-3 and PD-1 with mild and manageable immune-related side but functional improvement of T-cell anti-tumor immunity and other additional inhibitory receptors are “tier” 3 with limited or unknown functional change to solid tumors. Hereby, the index introduced to correct the

scoring system we proposed is essential as different “tiers” of inhibitory receptors contribute to T-cell exhaustion to different degrees. In the meanwhile, the functional quality of dominated and sub-dominated phenotypes of tumor infiltrating CD8 T-cells in each cancer should be further characterized to refine the scoring system as well. In addition, every inhibitory receptor in different cancer may contribute to the hyporesponsiveness of tumor infiltrating T cells to different degrees. Therefore, each cancer should have its own correction of individual inhibitory receptor in the evaluation of “immune-quality” in the tumor microenvironment.

3.2.6 Comparative analysis of T-cell differentiation between peripheral blood and tissues

During virus infection, T cells can proliferate and differentiate into multiple effector phenotypes to mediate immune responses for virus clearance and establishing long term protective immunity against re-infection in the future[224, 225]. T-cell differentiation involves 3 typical phases: clonal expansion, contraction of T-cell population and memory T-cell formation[224]. Within different phases, T-cell exhibits distinctive phenotypes and functions. In addition, recent studies have demonstrated that a special lineage of T-cell, known as tissue resident memory T cells, specifically resides in normal tissues rather than circulating in the peripheral has functionally and phenotypically distinctive characteristics, which indicates the importance of proper control when investigating T-cell differentiation from malignant human tissues[226]. Hereby, we compared the frequency of each stage of T-cell differentiation between the peripheral blood and cancer tissues from breast cancer individuals.

In parallel, the comparative analysis between peripheral blood and tissues was also conducted in benign breast lesion (fibroadenoma) individuals.

Figure 3-18: Expression analysis of T-cell differentiation between peripheral blood and breast tissues

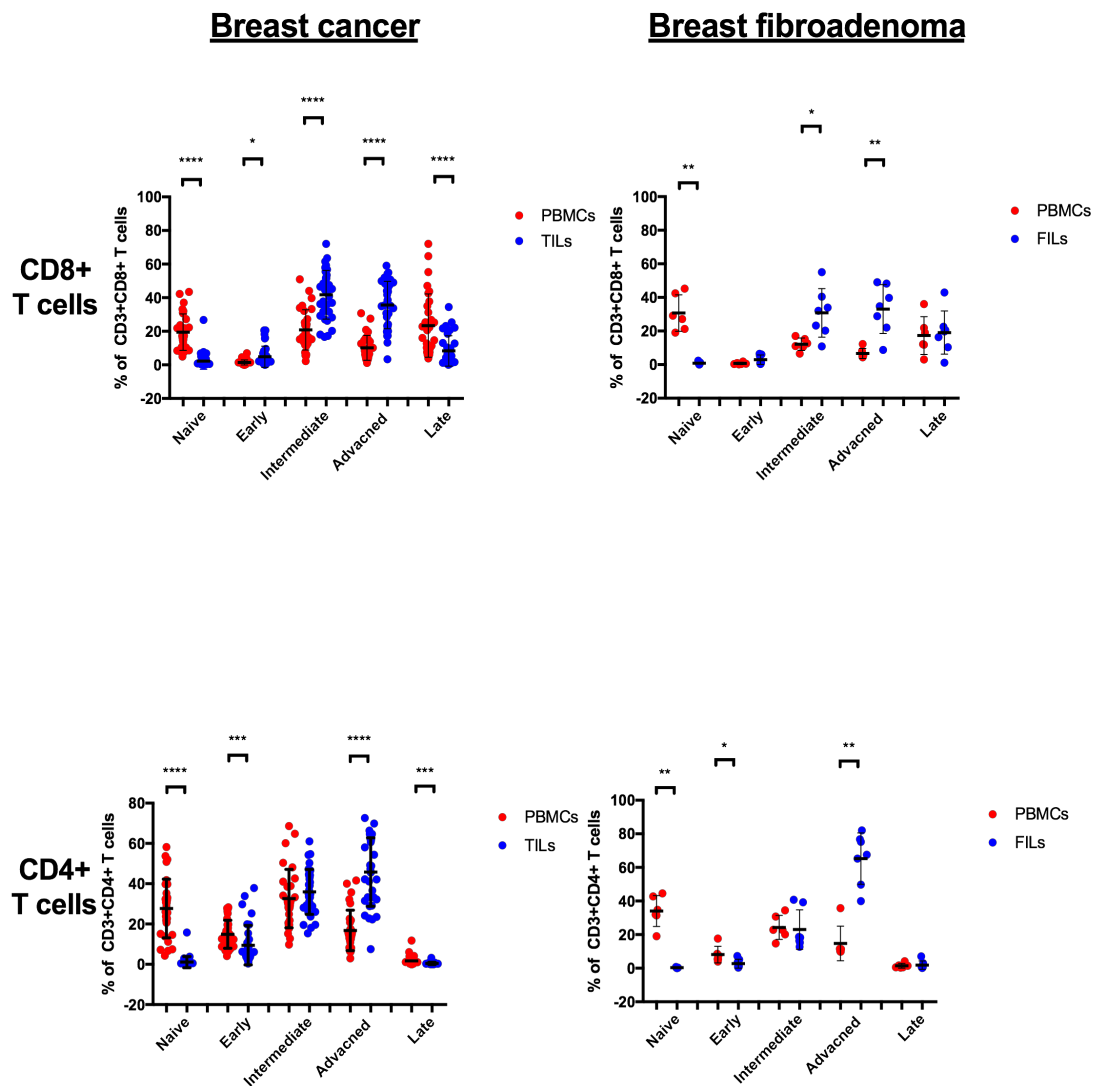


Figure 3-18: Expression analysis of 5 stages of T-cell differentiation subsets, CD27+CCR7+CD45RA+ (Naïve T-cell differentiation phenotype), CD27+CCR7+CD45RA- (early T-cell differentiation phenotype), CD27+CCR7-CD45RA- (intermediate T-cell differentiation phenotype), CD27-CCR7-CD45RA- (advanced T-cell differentiation phenotype) and CD27-CCR7-CD45RA+ (late T-cell differentiation phenotype) on CD4+ T cells and CD8+ T cells from fresh PBMCs, FILs and TILs in 43 breast cancer, 7 breast fibroadenoma individuals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney t-test). Top row: % in CD8+ T cells. Bottom row: % in CD4+ T cells.

In Figure 3-18, it was shown that in the frequency comparison of 5 T-cell differentiation stages in CD8 T cells, higher frequency of CD8+CD27+CCR7-CD45RA-(intermediate T-cell differentiation phenotype) and CD8+CD27-CCR7-CD45RA-(advanced T-cell differentiation phenotype) was observed in both benign and malignant breast tissues when compared with that in peripheral blood. In contrast, low frequency of CD8+CD27+CCR7+CD45RA+(Naïve T-cell differentiation phenotype) was found in both benign and malignant breast tissues when compared with that in peripheral blood. This indicates that CD8 T cells exhibit distinctive phenotypes between peripheral blood and tissues.

However, it was also found that the CD8+CD27-CCR7-CD45RA+(late T-cell differentiation phenotype) subset has a significantly lower frequency in breast cancer tissues but not in benign breast lesions when compared with that in peripheral blood. It suggests that this phenotype of CD8 T cells may have a distinctive function in the tumor microenvironment. CD8+CD27-CCR7-CD45RA+ has been described as a terminally differentiated T-cells phenotype[227]. But recent studies indicated that it may also have long-term memory T cells function[68]. In contrast, higher frequencies of naïve and early stages of CD4 T cells were observed in the peripheral blood when compared with tissues in both malignant and benign breast diseases. Similarly, lower frequency of late stage CD4 T cells was only observed in breast cancer tissues but not in benign breast lesions when compared with that in peripheral blood.

3.2.7 Expression analysis of inhibitory receptors in different T-cell differentiation stages between the peripheral and cancer tissues

During virus infection, having been primed by the antigens, naïve T-cell proliferates and differentiates into effector phenotypes to clear the pathogens[225, 228]. Most of the T cells are contracted after viral clearance and only a small percentage (5-10%) of T cells differentiate into memory phenotypes against re-infection[229]. With signal1 (antigen stimulation), signal2 (co-stimulation receptor-ligand interaction) and signal3 (inflammation environment), T cells could differentiate from naïve T cells to central memory T cells, transitional memory T cells, effector memory T cells and terminal effector T cells, which comprise a heterogeneous T-cell population in our body[230]. However, the dynamic expression of inhibitory receptors along with the process of T-cell differentiation has not yet been characterized. Understanding how T-cell differentiation impact the expression of inhibitory receptors may allow us to improve the blockade immunotherapies while decreasing the side effects to a manageable degree. 5 T-cell differentiation stages could be distinguished by the expression of CD27, CCR7 and CD45RA[231]. We extended the expression analysis of 2B4, PD-1 and KLRG-1 into different T-cell differentiation stages and investigated the influences T-cell differentiation may have on each inhibitory receptor in different tumor microenvironments. Taking the findings above together, we have characterized the expression of inhibitory receptors on this specific T-cell differentiation stage from multiple cancers.

Figure 3-19: Expression analysis of PD-1 and 2B4 on CD8+ T cells in different T-cell differentiation stages in HBV associated HCC individuals

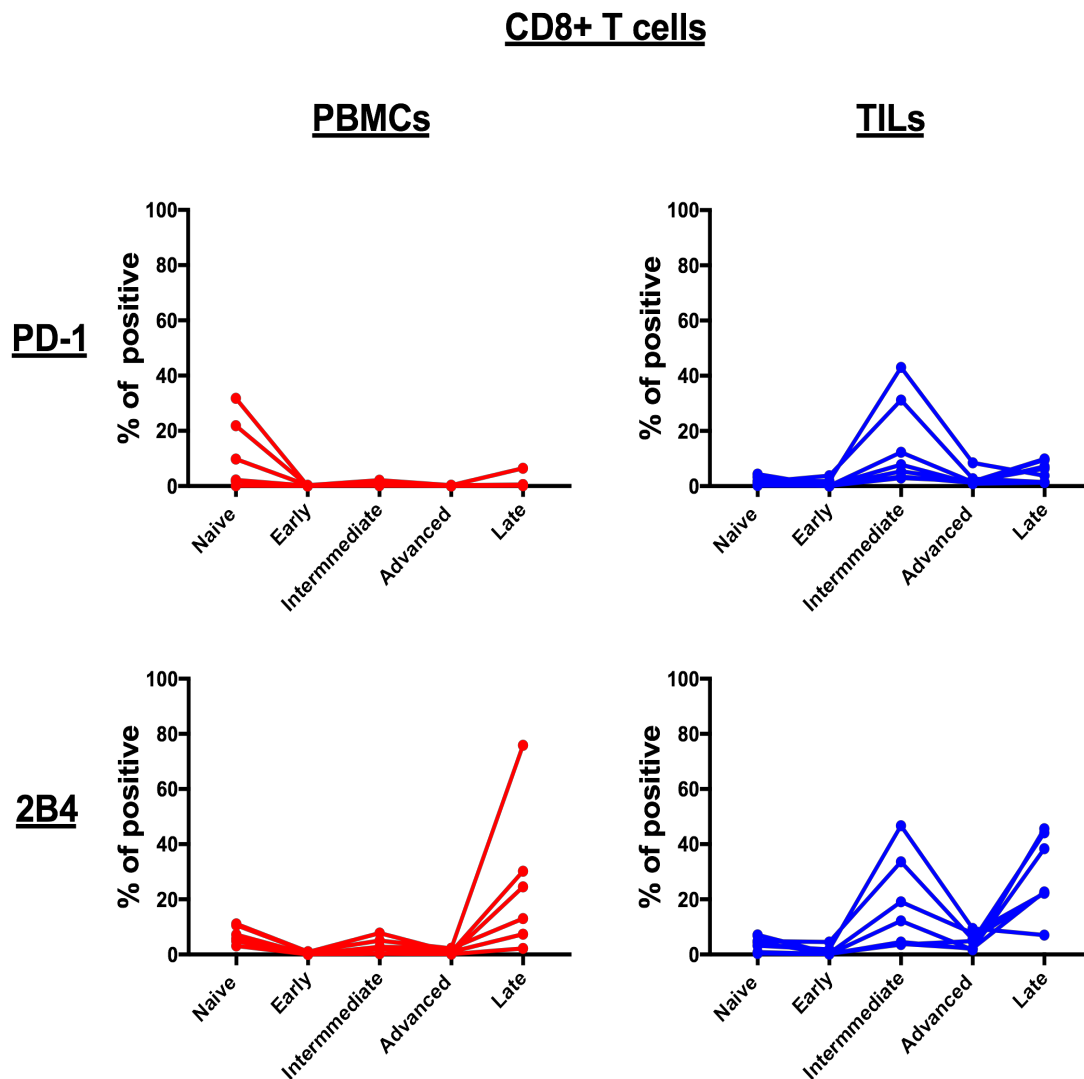


Figure 3-19: Expression analysis of PD-1 and 2B4 on CD8+ T cells in 5 different T-cell differentiation stages, CD27+CCR7+CD45RA+(Naïve T-cell differentiation phenotype), CD27+CCR7+CD45RA-(early T-cell differentiation phenotype), CD27+CCR7-CD45RA-(intermediate T-cell differentiation phenotype), CD27-CCR7-CD45RA-(advanced T-cell differentiation phenotype) and CD27-CCR7-CD45RA+(late T-cell differentiation phenotype). PBMCs and TILs were collected from 6 HBV associated HCC patients. Each line represents the frequency of inhibitory receptor in different T-cell differentiation stages from the individual patient. Top row: PD-1 expression in different T-cell differentiation stages. Bottom row: 2B4 expression in T-cell differentiation stages. Left graph: PD-1 and 2B4 expression in CD8+ T cells from PBMCs in % (red). Right graph PD-1 and 2B4 expression in CD8+ T cells from TILs in % (blue).

Figure 3-20: Expression analysis of PD-1 and KLRG-1 on CD8+ T cells in different T-cell differentiation stages in breast cancer individuals

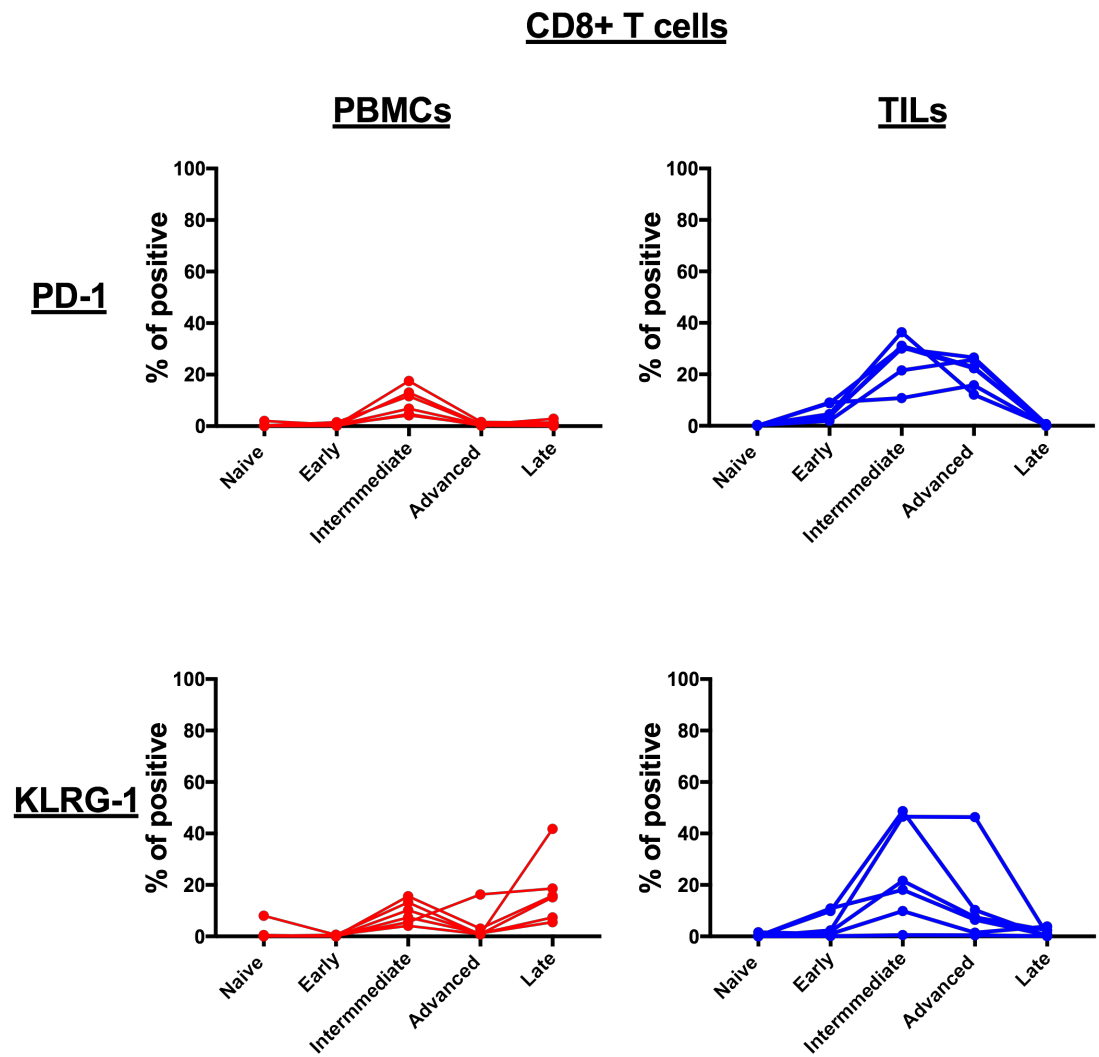


Figure 3-20: Expression analysis of PD-1 and 2B4 on CD8+ T cells in 5 different T-cell differentiation stages, CD27+CCR7+CD45RA+(Naïve T-cell differentiation phenotype), CD27+CCR7+CD45RA-(early T-cell differentiation phenotype), CD27+CCR7-CD45RA-(intermediate T-cell differentiation phenotype), CD27-CCR7-CD45RA-(advanced T-cell differentiation phenotype) and CD27-CCR7-CD45RA+(late T-cell differentiation phenotype). PBMCs and TILs were collected from 4 breast cancer patients. Each line represents the frequency of inhibitory receptor in different T-cell differentiation stages from the individual patient. Top row: PD-1 expression in different T-cell differentiation stages. Bottom row: 2B4 expression in T-cell differentiation stages. Left graph: PD-1 and 2B4 expression in CD8+ T cells from PBMCs in % (red). Right graph PD-1 and 2B4 expression in CD8+ T cells from TILs in % (blue).

Figure 3-21: Expression analysis of PD-1 and KLRG-1 on CD8+ T cells in different T-cell differentiation stages in colorectal cancer individuals

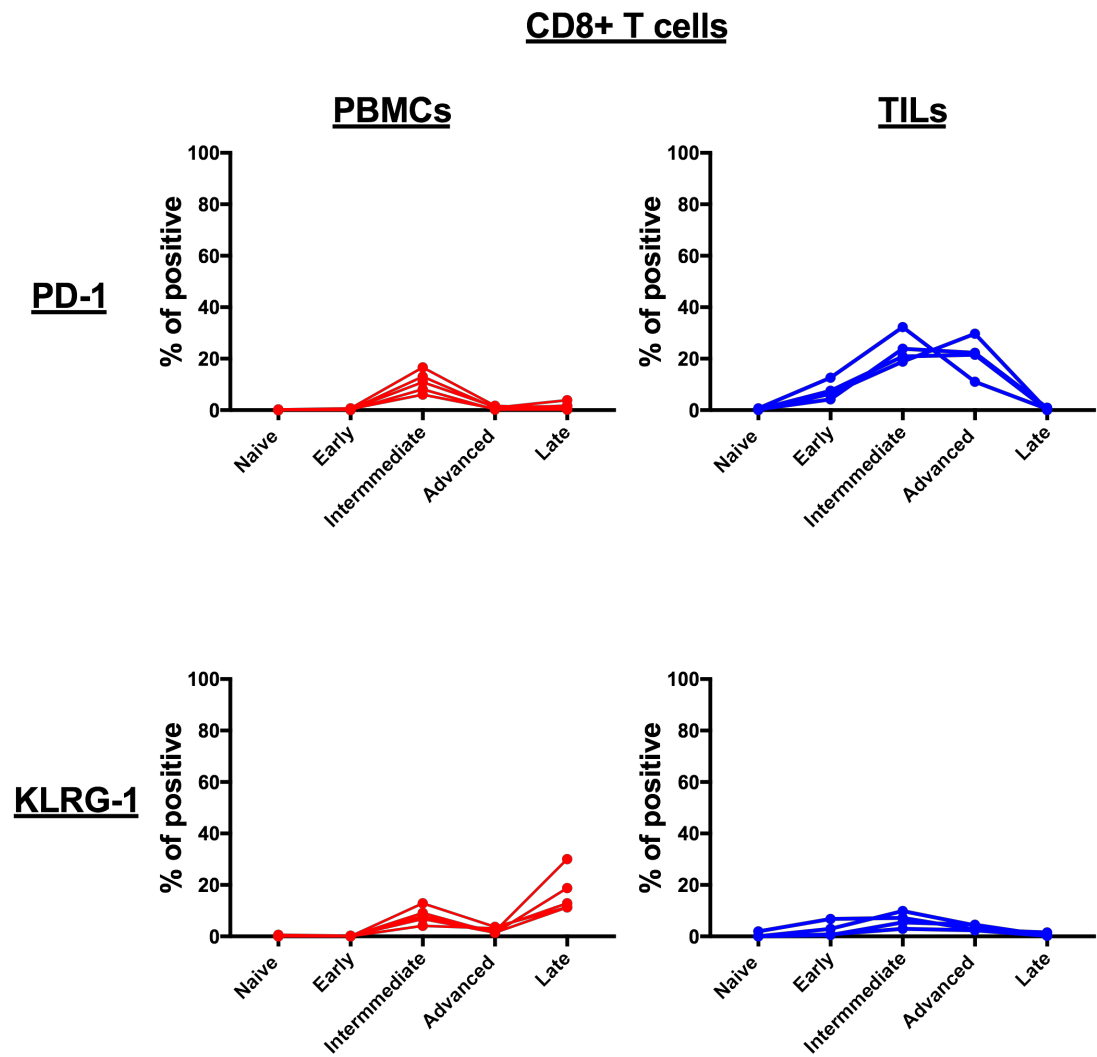


Figure 3-21: Expression analysis of PD-1 and 2B4 on CD8+ T cells in 5 different T-cell differentiation stages, CD27+CCR7+CD45RA+(Naïve T-cell differentiation phenotype), CD27+CCR7+CD45RA-(early T-cell differentiation phenotype), CD27+CCR7-CD45RA-(intermediate T-cell differentiation phenotype), CD27-CCR7-CD45RA-(advanced T-cell differentiation phenotype) and CD27-CCR7-CD45RA+(late T-cell differentiation phenotype). PBMCs and TILs were collected from 4 colorectal cancer patients. Each line represents the frequency of inhibitory receptor in different T-cell differentiation stages from the individual patient. Top row: PD-1 expression in different T-cell differentiation stages. Bottom row: 2B4 expression in T-cell differentiation stages. Left graph: PD-1 and 2B4 expression in CD8+ T cells from PBMCs in % (red). Right graph PD-1 and 2B4 expression in CD8+ T cells from TILs in % (blue).

In the first set of our experiments, we surprisingly found that the inhibitory receptor like BTLA has higher expression in the periphery compared with its counterparts in tumor tissues. Several studies have indicated that some inhibitory receptors are not just the markers for T-cells or NK cells inhibition. Meanwhile its expression also associates with T-cell differentiation and activation upon antigen stimulation[232]. BTLA expression in healthy donors has the higher expression in naïve T-cells phenotype and its expression declines in effector memory, central memory and terminally differentiated T-cells phenotypes[201].

Therefore in the final set of experiments in this chapter we aim to investigate the link of inhibitory receptors expression and T-cells differentiation. Differential expression of 2B4, KLRG-1 and PD-1 on CD8+ T cells was observed in 5 T-cell differentiation stages. Overall, in peripheral blood, PD-1 has higher expression in naïve CD8+ T-cell phenotype (20% on average) when compared with its expression in “early” stage, “intermediate” stage, “advanced” stage and “late” stage phenotypes in HBV associated HCC (Figure 3-19). Whereas, in the periphery of breast cancer individuals and colorectal cancer individuals, PD-1 was predominately up-regulated on CD8+ T cells in the “intermediate” stage phenotype (Figure 3-20, Figure 3-21). PD-1 expression on CD8+ TILs has similar expression pattern among HBV HCC individuals, breast cancer individuals and colorectal cancer individuals. On CD8+ TILs, PD-1 was progressively up-regulated in “intermediate” stage, while maintaining relatively low expression in the other 4 T-cell differentiation stages. In breast cancer individuals and

colorectal cancer individuals, KLRG-1 has higher expression on CD8+ T cells in “intermediate” stage and “late” stage phenotypes in the peripheral blood but its expression is only up-regulated on CD8+ TILs in “intermediate” stage phenotype at the tumor.

Even though the limited samples were available in this experiment, we still could perceive the expression of inhibitory receptors is linked with T-cell differentiation in different cancer patients. Interestingly, in non-chronically HBV infected breast cancer and colorectal cancer individuals, PD-1 predominately expressed on “intermediate” T-cell differentiation phenotype in both peripheral blood and tumor tissues. However, in HBV associated liver cancer individuals, PD-1 has higher expression in naïve T-cell differentiation phenotype in the peripheral but share similar expression in the tumor as that observed in breast cancer and colorectal cancer individuals. Our interpretation is that the persistent exposure to HBV virus may alter PD-1 expression in naïve phenotype in the blood.

KLRG-1 has a distinctive expression pattern along with different T-cell differentiation stages. Similar to PD-1 expression pattern, KLRG-1 mainly expresses on intermediate T-cell differentiation stage in the tumor in breast cancer and colorectal cancer individuals even its expression in colorectal cancer tissues is not lower than that observed in breast cancer. However, KLRG-1 expression has 2 “peaks” (“intermediate” and “late” stages), in 5 T-cell differentiation stages in the blood in both breast cancer and colorectal cancer

individuals. KLRG-1 has dual functionalities serving as inhibitory marker and T-cell differentiation marker. Hence, it is important to distinguish that if the up-regulation of KLRG-1 in different cancer tissues is a consequence of T-cell exhaustion or resident T-cell phenotype linking to T-cell differentiation.

3. 3 Discussion

In the first set of our analysis, we have observed the non-specific up-regulation of PD-1 in both malignant and benign tissues from HBV HCC individuals and breast cancer individuals respectively, when compared with its expression in the peripheral. This finding suggests that PD-1 has a physiological role in limiting T-cells responses towards tissue damage. However, PD-1 has higher expression on TILs when comparing with its expression on IHLs. This indicates that PD-1 blockade immunotherapy may apply to the treatment of HBV associated HCC. Multiple clinical trials of PD-1 inhibitor, either Nivolumab or Pembrolizumab in melanoma and other carcinomas such as NSCLC demonstrated clinical benefits to patients with variable ORR[54, 77, 80, 81, 197]. Ongoing clinical trials have already involved poor prognosis cancers like HCC with 18.9% ORR[233, 234].

In China, more than 50% of HCC cases are HBV associated[235]. On the one hand, chronic HBV infection and the emergence of cancer together contribute to dysfunctional T cells which worsens the reinvigoration of T-cell function to augment effective control of virus and cancers. On the other hand, if the blockade of the inhibitory pathway effect is very evident eliciting non-specific T-cell responses, it could exert a negative impact on HBV HCC patients as their liver function and liver damage tolerance are lower than non-virus infected HCC

individuals and other type of cancer individuals. Therefore, additional inhibitory pathways other than PD-1/PD-L1 might be promising targets to treat HBV HCC patients.

Of note, in acute HBV infected individuals, T cells are capable of mediating potent controls of virus, which is the reason why in non-immune compromised healthy adults, 95% of HBV infection would be resolves and self-limited within 6 months[236]. This leads to another consideration that PD-1 positive T-cell phenotypes from different types of tissues may have differential functional characteristics as PD-1 up-regulation was observed on both HBV associated malignant tissues and non-HBV benign liver tissues when compared with its counterparts in the blood. Further to this question, our data also demonstrated that in different stages of breast cancer individuals, no significant sole inhibitory receptor expression was detected within different stages. But in advanced stage of breast cancer individuals, its TILs may have poorer T-cell quality against cancer leading to failure in controlling tumor growth and metastasis. Therefore, it is of pivotal importance to further analyze functional characteristics of PD-1 positive T cells to fully characterize in T-cell exhaustion.

In breast cancer and breast fibroadenoma experiments, our data supports the proposed thought that different cancers may have different blockade regimens as Tim-3 has exclusively higher expression in malignant tissues when compared with the peripheral blood. This up-regulation was not observed in the breast fibroadenoma patients. Currently, PD-1 inhibitor has been approved to treat triple negative breast cancer individuals, one of the breast cancer types with

poorest prognosis[237]. Our data suggests that Tim-3 and PD-1 combinatorial blockade may improve anti-tumor efficacy and response rate, especially in PD-1 non-responders, in advanced breast cancer individuals.

Anti-CTLA-4 mAb, ipilimumab was the milestone of blockade immunotherapy and was the first inhibitory pathway medication approved by FDA and achieved positive benefits in advanced melanoma patients[238]. However, with more results revealed from multiple clinical trials covering different types of cancer, CTLA-4 inhibitor has demonstrated severe autoimmune adverse effects that have remarkable risks for patients[99, 104]. But those severe adverse effects were rare to observe in PD-1 inhibitors clinical trials, which broaden the extension of PD-1 usage in other cancers[75, 239, 240]. Our data in the analysis of CTLA-4 expression on global CD4 and CD8 T cells from tumor tissues imply that its expression is relatively low (mean expression is below 20% of TILs) when compared with that of PD-1. The effectiveness of CTLA-4 in melanoma treatment is correlated with the severity of autoimmunity, which indicates CTLA-4 is more likely a switch to release self-tolerance rather than specifically improve T-cell exhaustion caused by immunosuppressiveness in cancer[48]. Hence, lessons we have learnt from effects in CTLA-4 and PD-1 clinical trials are that (1) ideal sole inhibitory pathway blockade should have high specificity in resuscitating exhausted T cells caused by tumor microenvironment; (2) less and manageable immune-related adverse effects are expected particularly in chronically HBV/HCV infected liver cancer individuals. (3) PD-1 inhibitor combining with additional inhibitory pathway inhibitors may improve the response rate and sustain the anti-tumor responses. (4) an inhibitory receptor

based scoring system of T-cell exhaustion may be helpful to evaluate the quality and degrees of tumor infiltrating T cells[171]. More importantly it may provide crucial information for combinatorial blockade individually.

In our combinatorial expression analysis, we have observed the dominant phenotype of CD8+ tumor infiltrating T cells in different cancers. Of note, in HBV HCC patients, the dominant phenotype is CD3+CD8+2B4+KLRG-1+TIGIT+PD-1-CTLA-4. In liver cancer PD-1 inhibitor clinical trials, less than 20% patients response to PD-1 blockade. Our combinatorial expression analysis provide a rationale that KLRG-1, 2B4 and TIGIT might be the additional targets in treating liver cancer as the dominant phenotype of CD8 TILs in liver cancers.

Further, we have also detected that KLRG-1 expression is linked with T-cell differentiation in breast cancer and colorectal cancer tumor tissues. Similar to PD-1, KLRG-1 has a higher expression on intermediate stage of differentiated T cells, which is the effector T cell phenotype. Targeting this subset may achieve promising clinical benefits.

Our studies also compared other additional inhibitory receptors such as BTLA, CD160 and 2B4 with PD-1 and CTLA-4, which implies that they may not be suitable for as additional targets. Our data suggests that 2B4 predominately expressed on late stage T-cell in both peripheral blood and tumor. Although it has relatively higher expression in the intermediate T cell differentiation stage, its expression is generally high (mean expression is over 60% of CD8 T cells in

the blood) in the circulating blood in healthy donors and cancer patients. This indicates 2B4 inhibitory pathway is more likely a self-tolerance mechanism rather than pathological consequence caused by cancer.

CD160 was firstly proposed to be a promising target to block and so enable reinvigoration, of T-cell exhaustion in animal models[241]. However, when this inhibitory receptor was analyzed in multiple cancers in human, no significantly high expression was observed. Our data demonstrates that in 7 different types of cancer, CD160 expression on both CD8 and CD4 T cells is relatively low (mean expression is lower than 15% of T cells). Hence we postulated that CD160 might not be a good target in either sole inhibitory pathway blockade or combinatorial inhibitory blockade.

4 Chapter 4: Dissecting ligands expression from different tumor microenvironments

4.1 Introduction

Tumor microenvironment has become the key focus for cancer treatments and cancer immunotherapies as this type of tissue inciting molecular, cellular and physical changes[242]. It comprises multiple cell subsets such as cancer cells, cancer stromal cells, mesenchymal cells, endothelial cells and fibroblasts to evade immune surveillance[194]. Generally, tumor microenvironment impairs protective immunity against cancer from several aspects. Firstly, cancer cells, stromal cells and epithelial cells consist the unique tumor microenvironment structure that enables restricted entries of immune cells to penetrate into the solid tumor eliciting potent immune responses against cancer cells[216]. Secondly, the release of cytokines and chemokines as well as enzymes produced from tumor microenvironment, can lead effector cells to a hyporesponsive state and activate its apoptosis signalling[215]. Thirdly, tumor microenvironment could dampen the presentation of tumor associated antigens that can be recognized by tumor infiltrating T cells via direct and indirectly influences to antigen presenting cells[171].

In the inhibitory pathway blockade therapy, PD-1/PD-L1 axis has achieved promising benefits in multiple cancer including melanoma, non-small cell lung cancer, bladder cancer, gastric cancer, renal cancer, triple negative breast cancer and liver cancer[48, 69, 240, 243]. It is also indicated that PD-L1, as one of the ligands of PD-1, may serve as a predictive marker in cancer

immunotherapy[244]. However, with more results published from clinical trials in multiple cancers, it indicates that PD-L1 as a potential predictive biomarker could rule out a certain proportion of patients who may benefit from PD-1/PD-L1 blockade immunotherapy. For instance, in metastatic melanoma patients, about 19% of PD-L1 negative patients also responded to the PD-1 blockade immunotherapy[48]. In addition, other inhibitory pathways in the scenario of T-cells mediated anti-tumor response may also improve cancer patients' prognosis and overall survival[171]. In the data that was shown in last chapter, we have observed the heterogeneous expression of PD-1 on CD8 TILs in breast cancer had quite a wide range (frequency of PD-1 from 0.2% of CD8 TILs to 80% of CD8 TILs in breast cancer). Hence, we aim to test that whether PD-1 expression on TILs correlates with the expression of one of its ligands, PD-L1 in tumor microenvironment. Clinical trials of PD-1 inhibitors were approved in triple negative breast cancer but not in other pathological types[245]. We recruited a total of 39 primary breast cancer individuals (only 1 out 39 is triple negative breast cancer) for this correlation study to provide additional information about PD-1/PD-L1 inhibitory pathway in non-triple negative breast cancer. To our knowledge, this is the first study that investigates the correlation between PD-1 expression on TILs and PD-L1 expression in tumor microenvironment on a specific subtype of breast cancer cohort. In the meantime, we have also conducted the same set of experiments to quantify the expression of Galectin-9, the ligand of Tim-3 and e-cadherin, the ligand of KLRG-1 simultaneously.

Previous quantification of ligands expression was predominately on immunohistochemistry (IHC)-based analysis. However, this classical cellular

expression analysis has several unsolved issues that may mask the correlation between inhibitory receptor expression and its ligands expression. With the development of fluorescent microscopy and antibodies, confocal microscopy may yield more detailed and accurate information in the quantification of proteins from tissues. Firstly, confocal microscopy allows us to more precisely scan the same thickness of samples to limit the bias that introduced from IHC. Secondly, confocal microscopy quantifies the ligands expression by the mean or median fluorescent intensity of the samples, which is more sensitive than IHC that semiquantifies the expression by the contrast of the dyes. Thirdly, confocal microscopy provides superior information of the localization of ligands expression in tumor microenvironments that may have pivotal role in the prediction and administration of relevant blockade immunotherapies.

Aims of the study in this chapter:

- 1: Characterizing the expression pattern of PD-L1, Galectin-9 and e-cadherin from 39 paired breast cancer individuals.
- 2: Quantifying the expression of PD-L1, Galectin-9 and e-cadherin from 39 paired breast cancer individuals in disease stage based manner.
- 3: Identifying the correlation between the expression of PD-L1, Galectin-9 and e-cadherin and its inhibitory receptors on CD4 and CD8 T cells from 39 paired breast cancer individuals.

4.2 Results

4.2.1 Optimization of the paraffin embedded human sample treatments for ligand expression analysis by confocal microscopy

Paraffin embedded human samples enable researchers to perform cellular expression assays and quantify protein expression at day-to-day clinical work especially in pathological test by immunohistochemistry[246]. More importantly, it maintains morphologic features of the samples and certain gene expression profiles if appropriate antigen retrieval and sample treatments were conducted. Most of the hospital based research institutes have established bio-banks containing paraffin embedded human samples, which allows the retrospective studies and validation of novel research discoveries. To analyze the ligands expression in human tumor microenvironment, we have preserved fresh surgical human tumor samples by formalin fixation and further made paraffin embedded blocks from 39 breast cancers individuals. All those patients have paired *ex vivo* flow cytometric data.

However, in the fluorescence-based studies such as protein expression analysis by fluorescent microscopy or confocal microscopy, the laser power introduced significant amount of auto-fluorescence on paraffin embedded tissues including human lung tissues, liver tissues, pancreatic tissues and kidney tissues, which mask the visualization of protein expression in tissues and insert false positivity in the quantification protein expression[247]. Several factors contribute to the auto-fluorescence of human paraffin embedded samples. 1: human tissues

consist of natural substances such as flavins and porphyrin in live human tissues that could generate the natural “background” under the excitation of lasers; 2: Under excitation from 360nm to 647nm wavelength from lasers, strong auto-fluorescence caused by a common products-lipofuscins that could be detected in a wide range of cell types, especially in vessel rich organs such as liver, kidney and spleen. 3: Reagents such as aldehyde, used in the sample fixation and antigen retrieval, could cause auto-fluorescence.

Multiple methods to tackle auto-fluorescence in human paraffin embedded sections have been proposed and demonstrated variable effects. Generally it consists of 2 categorical treatments in diminishing auto-fluorescence in human paraffin embedded samples, physical treatments and chemical treatments. The physical treatments comprise heat-steaming and photobleaching. Meanwhile, chemical treatments consist of variable quenchers including Sudan Black , TrueBlack and sodium borohydride, etc. At the very beginning of our ligands expression analysis, we have compared several methods including heat-steaming, sodium borohydride treatment and trueblack treatment on our samples to investigate which treatment is best in reducing auto-fluorescence.

Figure 4-1: Auto-fluorescence detected on human liver cancer paraffin embedded sample in green, blue and red channels on Zeiss 880 confocal microscopy

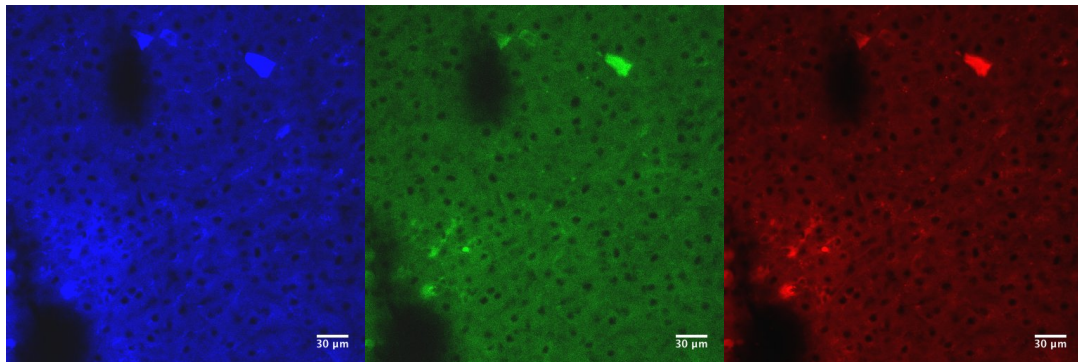
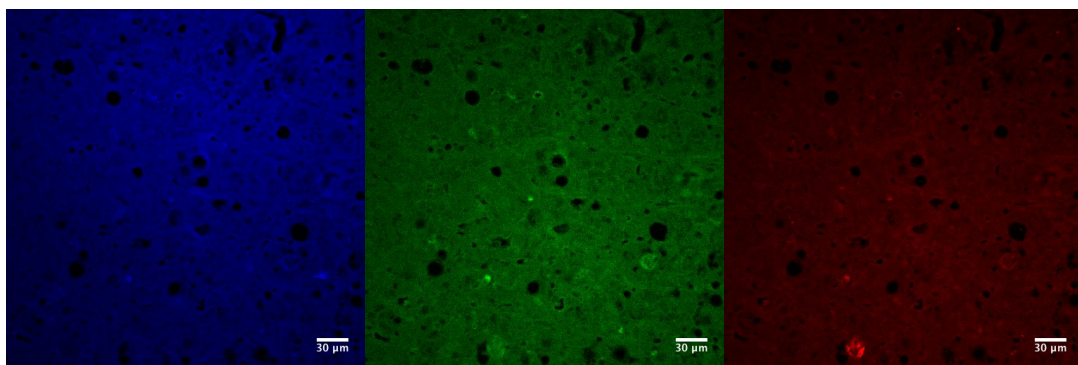


Figure 4-1: one HBV associated HCC tumor FFPE block sliced at 20 microns and underwent antigen retrieval. Graphs acquired from Zeiss 880 confocal microscopy on 40X2.5 oil lens excited by blue (561nm)-left, green (488nm)-middle and far red (633nm)-right lasers depicted at 512X512 pixels.

Figure 4-2: Sodium borohydride treatment cannot significantly reduce auto-fluorescence on human liver cancer paraffin embedded sections

Non treatment



Sodium borohydride treatment

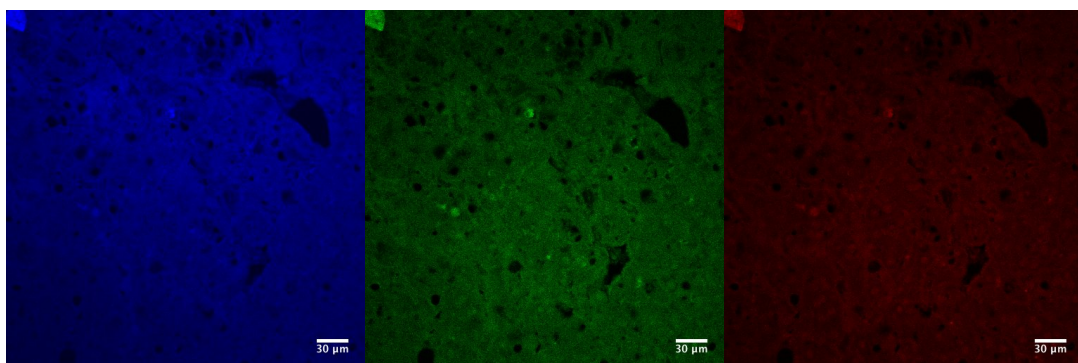


Figure 4-2: one HBV associated HCC tumor FFPE block sliced at 20 microns and underwent antigen retrieval with or without sodium borohydride treatment. Graphs acquired from Zeiss 880 confocal microscopy on 40X2.5 oil lens excited by blue (561nm)-left, green (488nm)-middle and far red (633nm)-right lasers depicted at 512X512 pixels.

Figure4-3: Trueblack quencher treatment significantly reduce auto-fluorescence on red channel but not on blue and green channel

Non trueblack quencher treatment

Trueblack quencher treatment

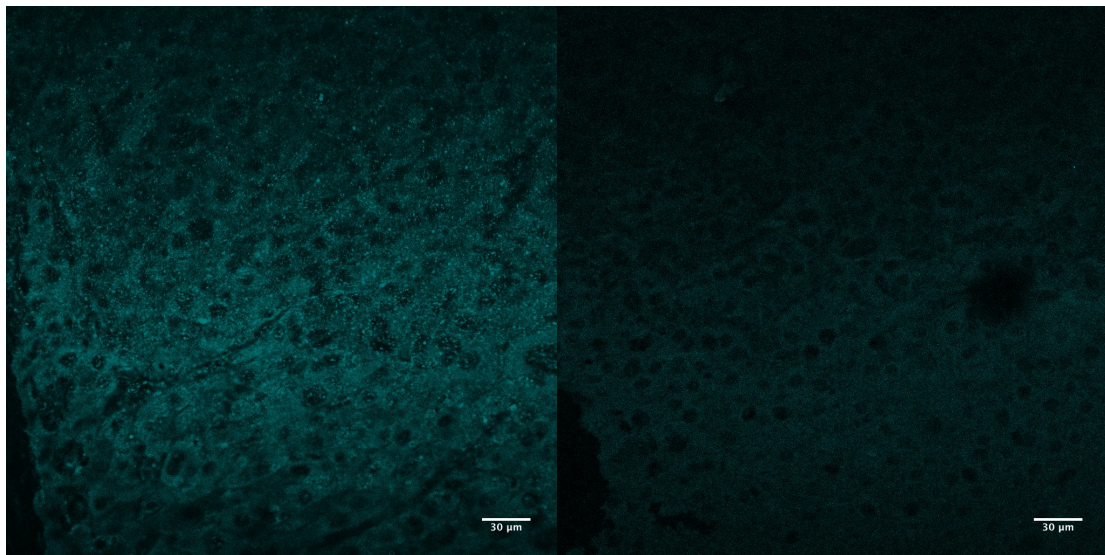


Figure 4-3: one HBV associated HCC tumor FFPE block sliced at 20 microns and underwent antigen retrieval. Graphs acquired from Zeiss 880 confocal microscopy on 40X2.5 oil lens excited by far red (633nm) lasers depicted at 512X512 pixels. Left was not treated with trueblack quencher. Right graph was treated with trueblack quencher to reduce auto-fluorescence.

Figure 4-4: Comparison of auto-fluorescence reduction by trueblack quencher in paired liver cancer and breast cancer individuals

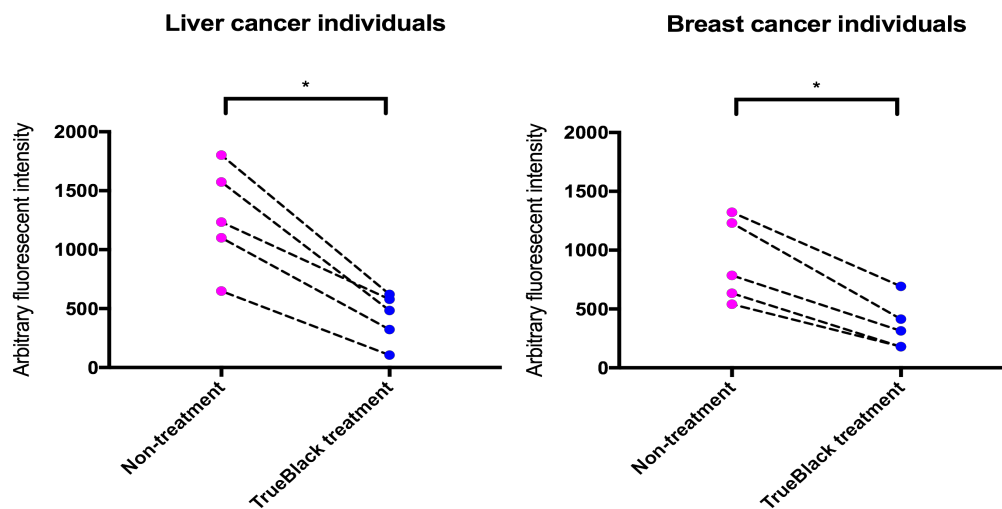


Figure 4-4: Quantification of fluorescence intensity in red channel (647nm) on Zeiss 880 confocal microscopy in 5 paired paraffin embedded sections from liver cancer individuals and breast cancer individuals respectively with or without treatment of trueblack quencher. In both types of cancers, trueblack significantly reduce auto-fluorescence in far red channel. Paired t test was applied to analyze the significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Wilcoxon t-test).

From results in Figure 4-1, 4-2, it was shown that paraffin embedded human tumor samples has considerable auto-fluorescence when conducting confocal microscopy to quantify the ligands expression in tumor microenvironments. This would be an obstacle that may hamper studies in the quantification of protein expression. From the published and newly identified methods, our results suggested that the sodium borohydride could not diminish auto-fluorescence on FFPE tumor samples from HBV associated HCC individuals. This is probably due to the unique composition of the liver that is a vessel-rich organ with elastics and collagens and synthesizing multiple enzymes[204]. Elastics, collagens as well as lipofuscins together contribute to the auto-fluorescence when excited by the lasers.

In figure 4-3, 4-4, it was found that the newly identified chemical quencher, Trueblack enables the reduction of auto-fluorescence in far red channel from human tumor tissues in confocal microscopy to a relatively low level which has little effects on the quantification of ligands expression as well as the quantification of expression pattern. Although co-localization in this setting could not be available technically, we still could conduct our correlation study between inhibitory receptors expression and ligands expression by one channel on confocal microscopy.

4.2.2 Differential ligands expression pattern in tumor microenvironment

Previous clinical trials in melanoma patients treated with PD-1 blocking antibody, Nivolumab suggested the differential expression patterns of PD-L1 observed from IHC in tumor samples[48, 248]. Specifically it has cytoplasmic, membranous and none expression patterns in tumor samples from those melanoma individuals[48]. Some results of Nivolumab clinical trials in treating melanoma individuals indicate that before treatment, PD-L1 membranous expression of tumor cells from the recruited melanoma individuals positively correlates with the overall response rate (ORR) after treatment[56, 69].

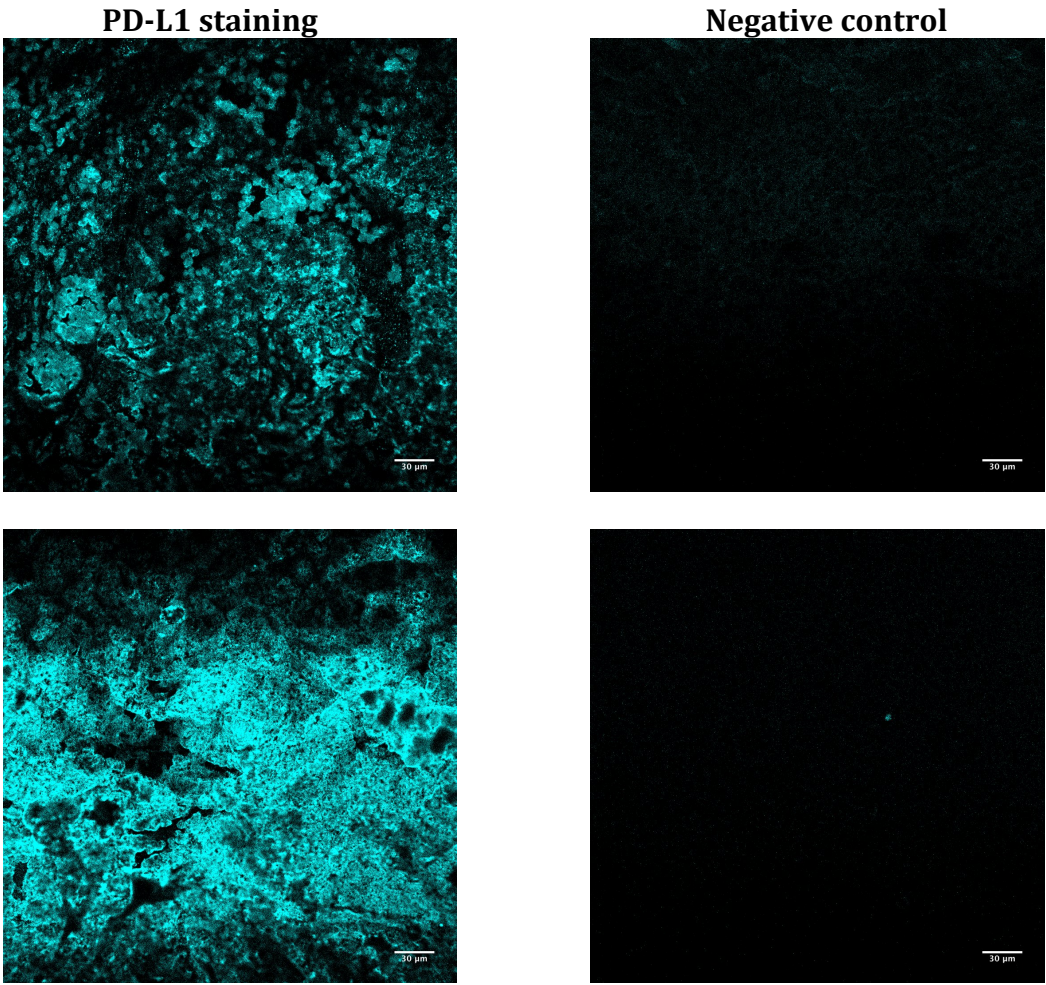
However, in a phase III Nivolumab clinical trial in treating advanced melanoma individuals, it was found that all subgroups of patients benefit from the Nivolumab treatment regarding its PD-L1 expression status[73, 78, 240]. Those results may question whether PD-L1 could be a biomarker to guide the application of PD-1 inhibitors. Furthermore, the revealed 3 expression patterns may also correlate with PD-1/PD-L1 blockade efficacy and clinical prognosis after treatment. Note that the expression pattern of PD-L1 in the subtype of

breast cancer is yet to be characterized. Based on the controversial results from current clinical trials, we simultaneously investigated the PD-L1, galectin-9 and e-cadherin expression patterns in 39 breast cancer individuals.

4.2.2.1 PD-L1 has membranous, cytoplasmic expression patterns in breast cancer tumor microenvironment

Figure 4-5: cytoplasmic, membranous and none expression of PD-L1 from breast cancer individuals

A: Cytoplasmic expression of PD-L1 from 2 breast cancer individuals



B: Membranous expression of PD-L1 from 2 breast cancer individuals

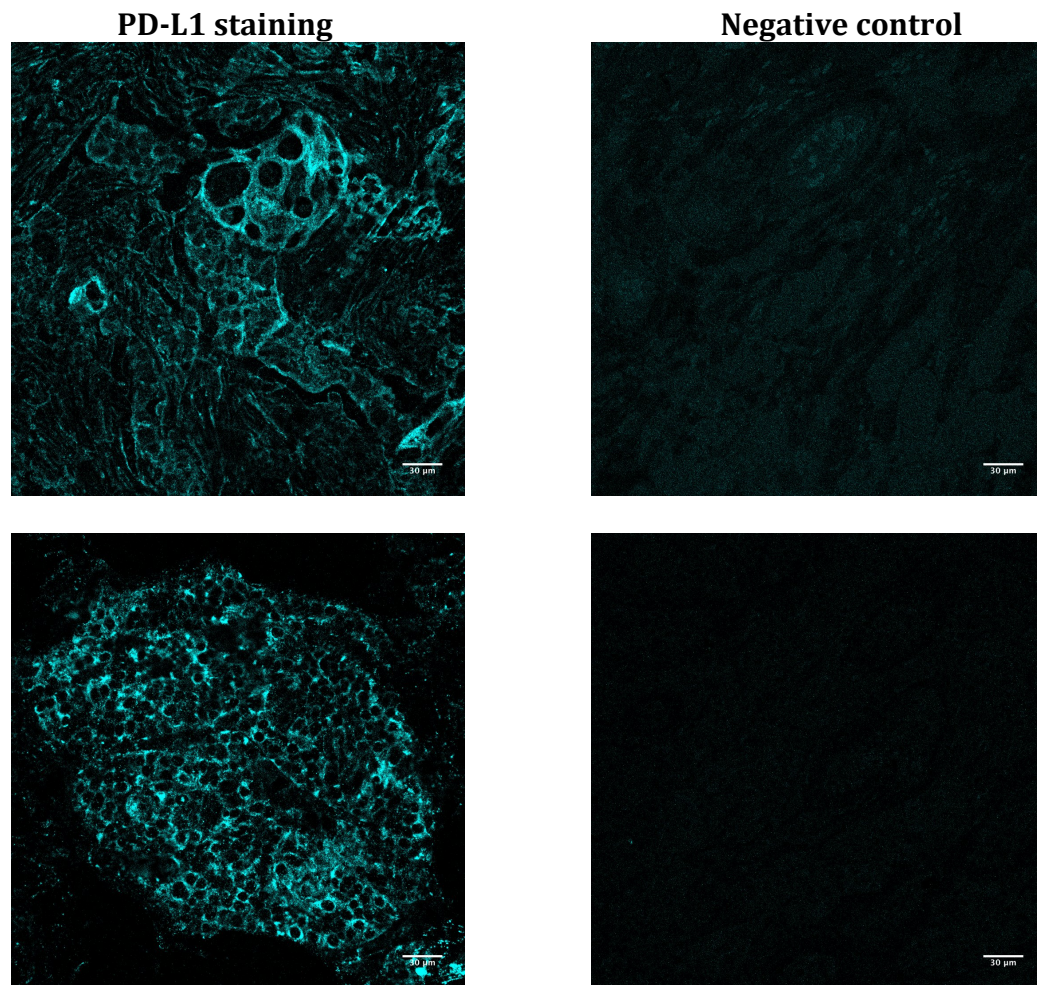


Figure 4-5: Expression of PD-L1 in FFPE sections sliced at 20 microns from breast cancer individuals, primary antibody: rabbit anti-human PD-L1, secondary antibody: goat anti-rabbit abbeviator red. Graphs were acquired by Zeiss 880 confocal microscopy in far red channel at 6.5 laser power, using 40X2.5 oil lens, scanning at 512X512 pixels. A: cytoplasmic expression of PD-L1 in 2 breast cancer tumor samples, left 2 graphs are positive staining, right 2 graphs are negative controls; B: membranous expression of PD-L1 in 2 breast cancer tumor samples, left 2 graphs are positive staining, right 2 graphs are negative controls; C: non expression of PD-L1 in 2 breast cancer tumor samples, left 2 graphs are positive staining, right 2 graphs are negative controls; Negative controls are secondary antibody staining only but with same treatment.

4.2.2.2 e-cadherin has membranous expression pattern on breast cancer tumor microenvironment

Figure 4-6: membranous and none expression of e-cadherin from breast cancer individuals

A: Membranous expression of e-cadherin from 2 breast cancer individuals

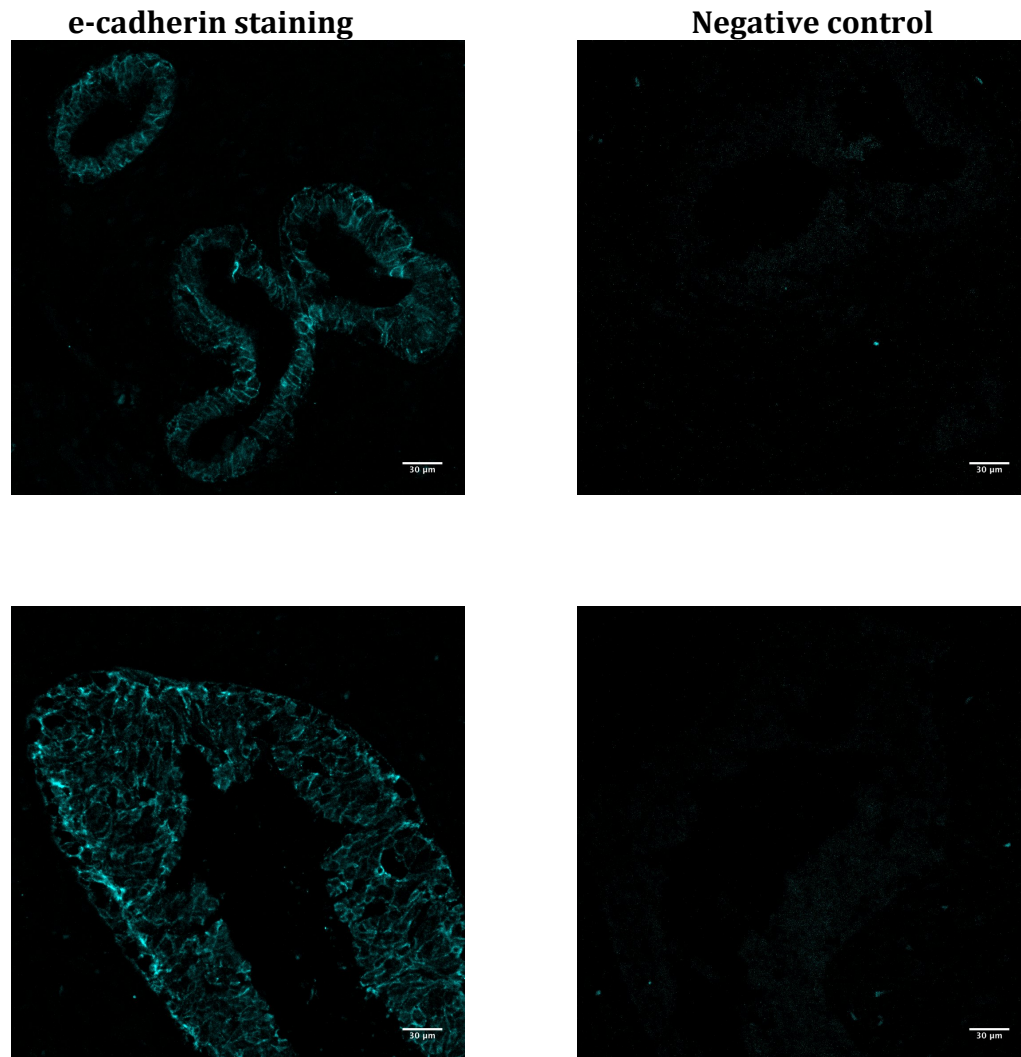


Figure 4-6: Expression of e-cadherin in FFPE sections sliced at 20 microns from breast cancer individuals, primary antibody: mouse anti-human e-cadherin, secondary antibody: goat anti-mouse abbeviator red. Graphs were acquired by Zeiss 880 confocal microscopy in far red channel at 6.5 laser power, using 40X2.5 oil lens, scanning at 1024X1024pixels. A: cytoplasmic expression of e-cadherin in 2 breast cancer tumor samples, left 2 graphs are positive staining, right 2 graphs are negative controls; B: non expression of e-cadherin in 2 breast cancer tumor samples, left 2 graphs are positive staining, right 2 graphs are negative controls; Negative controls are secondary antibody staining only but with same treatment

4.2.2.3 Galectin-9 has membranous pattern on breast cancer tumor microenvironment

Figure 4-7: membranous and none expression of Galectin-9 from breast cancer individuals

A: Membranous expression of galectin-9 from 2 breast cancer individuals

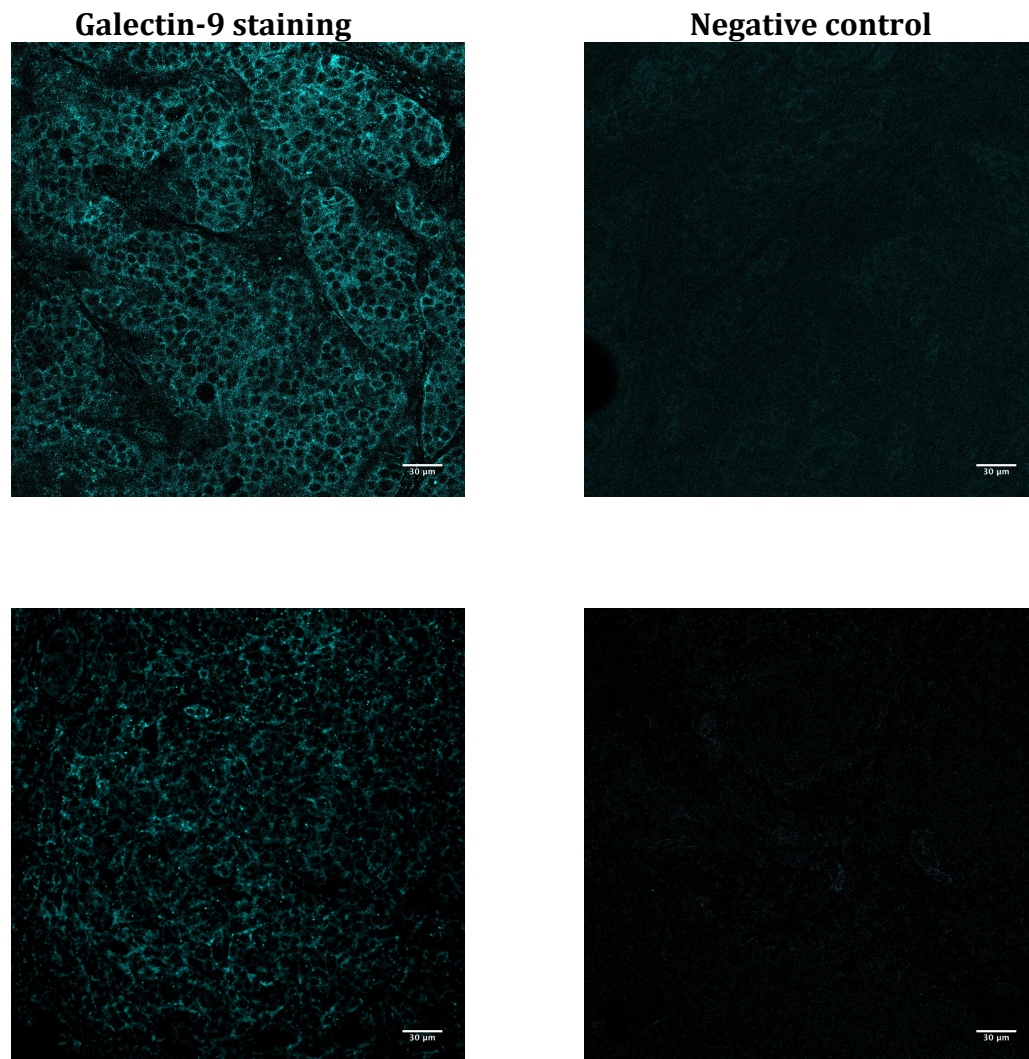


Figure 4-7: Expression of Galectin-9 in FFPE sections sliced at 20 microns from breast cancer individuals, primary antibody: rabbit anti-human Galectin-9, secondary antibody: goat anti-rabbit abbeviator red. Graphs were acquired by Zeiss 880 confocal microscopy in far red channel at 6.5 laser power, using 40X2.5 oil lens, scanning at 1024X1024 pixels. A: membranous expression of Galectin-9 in 2 breast cancer tumor samples, left 2 graphs are positive staining, right 2 graphs are negative controls; B: non expression of galectin-9 in 2 breast cancer tumor samples, left 2 graphs are positive staining, right 2 graphs are negative controls; Negative controls are secondary antibody staining only but with same treatment.

In conclusion, differential expression patterns of PD-L1, Galectin-9 and e-cadherin were observed in breast cancer FFPE sections by confocal microscopy. 2 expression patterns (cytoplasmic and membranous expression patterns) of PD-L1 were detected in our breast cancer cohort, which is similar to what has been found in melanoma patients by IHC assays from several clinical trials. Next question is that if 2 positive (cytoplasmic and membranous) expression patterns of PD-L1 in tumor exert different impacts on PD-1/PD-L1 axis inhibition therapy. The differential expression patterns may also associate with disease stages and response rate when using PD-1 blockade therapy. In addition, as we have observed 2 PD-L1 expression patterns in stage I, stage II and stage III breast cancers, in clinical trials of Nivolumab and Pembrolizumab on other cancers, it may be worth checking in PD-L1+ tumor patients by IHC system, whether the responders and non-responders have preferential PD-L1 expression patterns.

However, we have also noticed that several factors may affect our interpretation.

1: We could only use a channel to quantify the expression of sole ligand expression due to autofluorescence. This limits the co-expression analysis of multiple ligands and the identification of specific subsets in tissues. 2: In some PD-L1 high expression samples, we could not distinguish dual expression patterns (cytoplasmic + membranous expression) of PD-L1 from sole cytoplasmic expression pattern. 3: the arbitrary fluorescent intensity has high sensitivity for quantitative analysis but lack of reliable cut-off value to define PD-L1 positivity, as a clinical reference. However, it may serve as an additional method along with IHC assays to analyze PD-L1 and other ligands expression

when conducting clinical trials of blockade drugs in multiple cancers since we have established the quantification analysis of ligand expression in human FFPE samples by confocal microscopy. The recruitment of sufficient benign tissues to calculate cut-off value of each ligand expression and validation of specific clones of antibodies used in the staining are the prerequisites and essential for large cohort studies.

Interestingly, apart from PD-L1 expression analysis, we have also simultaneously measured 2 ligands from additional inhibitor pathways, the Tim-3/Galectin-9 axis and KLRG-1/e-cadherin axis. Notably, Galectin-9 is not the only ligand for Tim-3, Neither for e-cadherin to KLRG-1. Our data suggests that unlike PD-L1, the Galectin-9 and e-cadherin expression in breast cancer tumor microenvironments are preferentially expressed in membranous pattern, where no cytoplasmic expression pattern was observed in our cohort. This indicates that compared to PD-1/PD-L1 inhibition of tumor infiltrating T cells; Tim-3/galectin-9 and KLRG-1/e-cadherin inhibitory pathways may contribute to T-cell exhaustion by a different mechanism and magnitude. Especially KLRG-1 also serves as a marker of T-cell differentiation[224, 231]. Meanwhile, the development of those additional inhibitory pathways such as KLRG-1/e-cadherin should also consider the effects on the establishment of autoimmunity and immunopathology[194]. Last but not least, in different cancers, the analysis of inhibitory receptors on effector cells and the expression of ligands expression should be linked to characterize its potential in the ultimate goaling-sustaining potent anti-tumor responses. In all, those findings may serve as useful information when conducting sole inhibitory pathway blockade and further

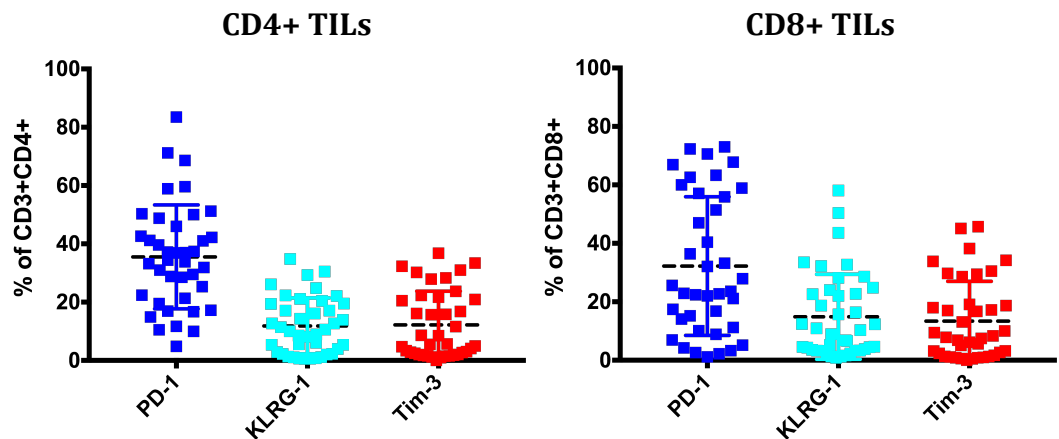
combinatorial blockade immunotherapy. Due to the technical limitation, we unfortunately could not perform co-localization of multiple ligands expression in tumor microenvironment, but this would be a valuable opportunity for future investigation.

4.2.3 Identical hierarchical expression pattern observed between inhibitory receptors on tumor infiltrating T cells and its ligands expression in tumor microenvironment

In the 39 breast cancer individual samples on which ligands expression analysis was performed, we have also conducted the *ex vivo* phenotypic analysis in the expression of inhibitory receptors on tumor infiltrating T cells. In Figure 4-8, we firstly analyzed the hierarchy of ligands expression compared with the hierarchy of inhibitory receptors they interact with. This would allow us to analyze that which currently identified ligand is dominant in tumor microenvironment so contributing to the inhibitory signalling and whether the same expression hierarchy would be observed between inhibitory receptors and its ligands. Furthermore, we stratified 39 breast cancer individuals into 3 different subgroups based on their TNM classification. In this set of comparisons, we could investigate if any differential expression of 3 ligands could be observed among breast fibroadenoma and different stages of breast cancer individuals.

Figure 4-8: Hierarchy of inhibitory receptors expression on TILs and hierarchy of ligands expression in tumor microenvironment

A: PD-1 dominates the hierarchy of 3 inhibitory receptors on T cells from breast cancer individuals



B: PD-L1 dominates 3 ligands expression in breast cancer tumor microenvironment

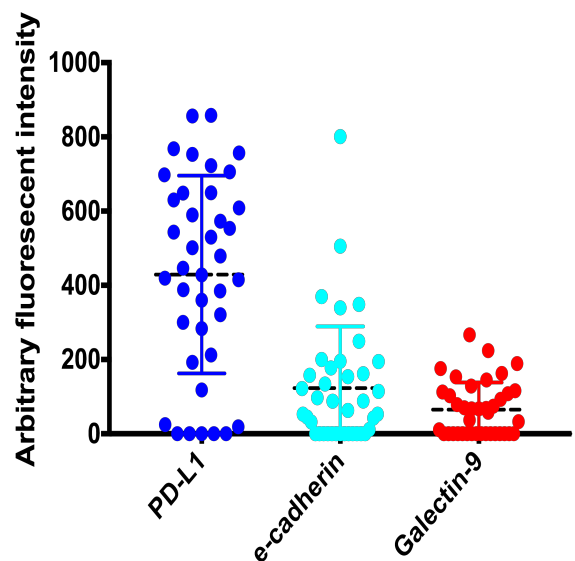
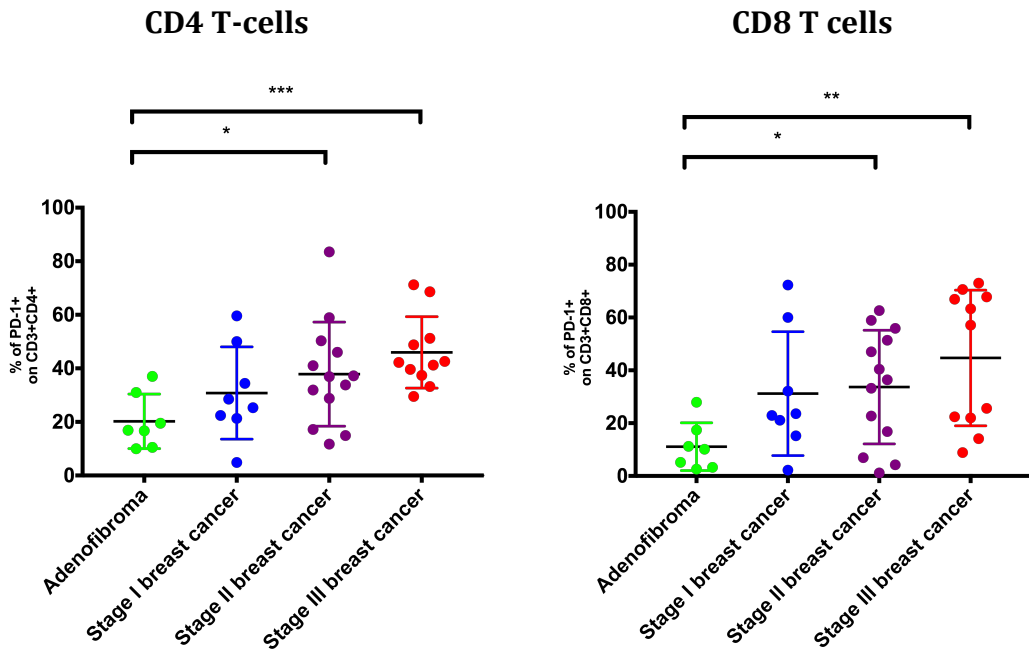


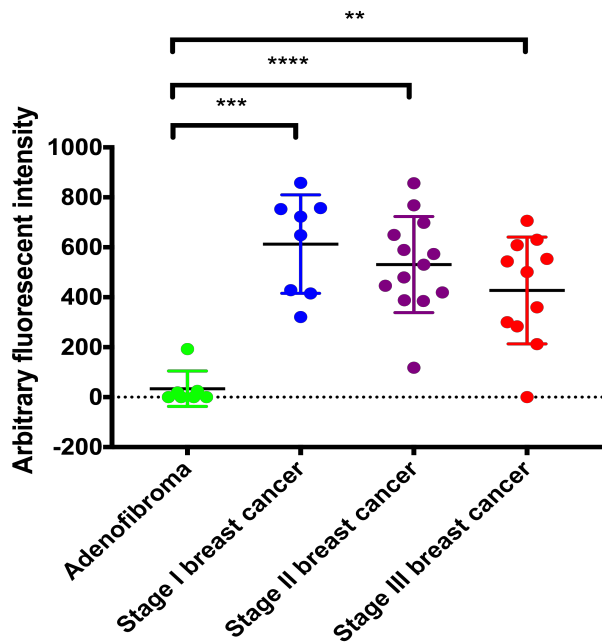
Figure 4-8: Expression analysis between inhibitory receptors on T cells and ligands expression in tumor microenvironment from 39 paired breast cancer individuals, panel A: PD-1, KLRG-1 and Tim-3 expression of on CD4+ TILs (left graph) and on CD8+ TILs (right graph) in (%); Panel B: Ligands expression of PD-L1, e-cadherin and Galectin-9 from human FFPE slides. Data acquired by confocal microscopy and analyzed in Fiji software, mean fluorescent intensity was measured in positive staining slide. Arbitrary fluorescent intensity was obtained by subtracted the mean fluorescent intensity of negative controls from the mean fluorescent intensity of positive staining slides.

Figure 4-9: Stage based expression analysis of inhibitory receptors on T cells and ligands expression in tissue microenvironment from paired breast fibroadenoma and breast cancer individuals

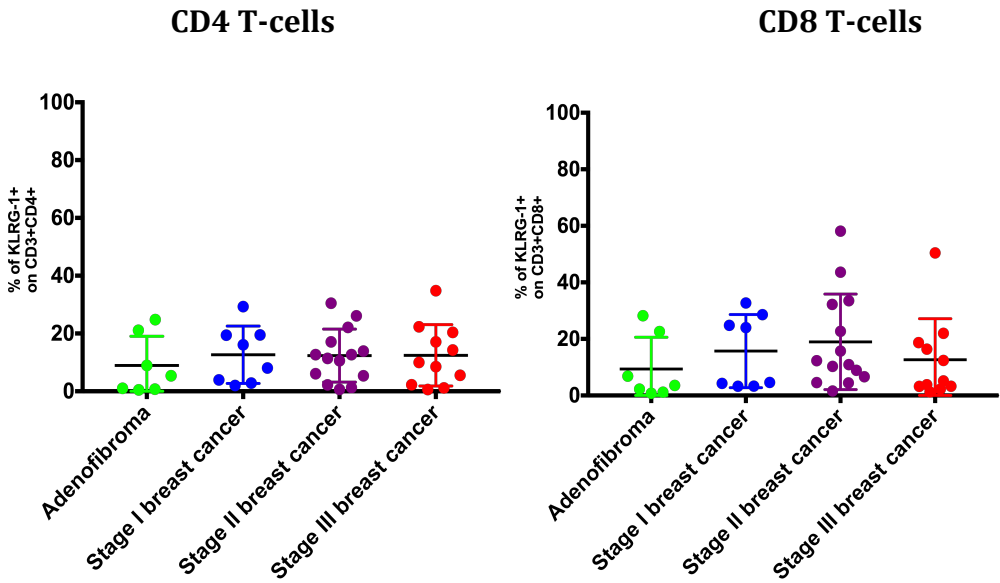
A: Differential PD-1 expression on CD4 and CD8 T cells in different breast disease stages



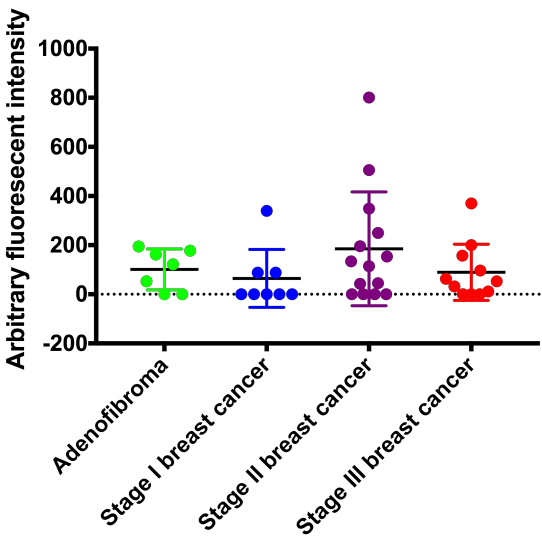
B: Differential PD-L1 expression in tissues from different breast disease stages



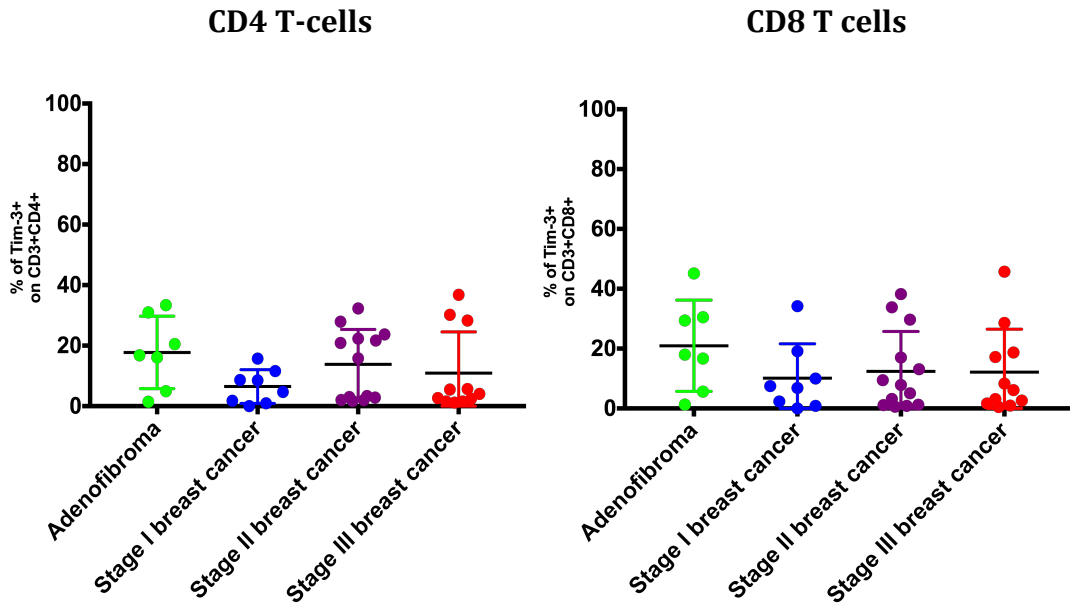
C: No differential KLRG-1 expression on CD4 and CD8 T cells in different breast disease stages



D: No differential e-cadherin expression in tissues from different breast disease stages



E: No differential Tim-3 expression in tissues from different breast disease stages



F: No differential Galectin-9 expression in tissues from different breast disease stages

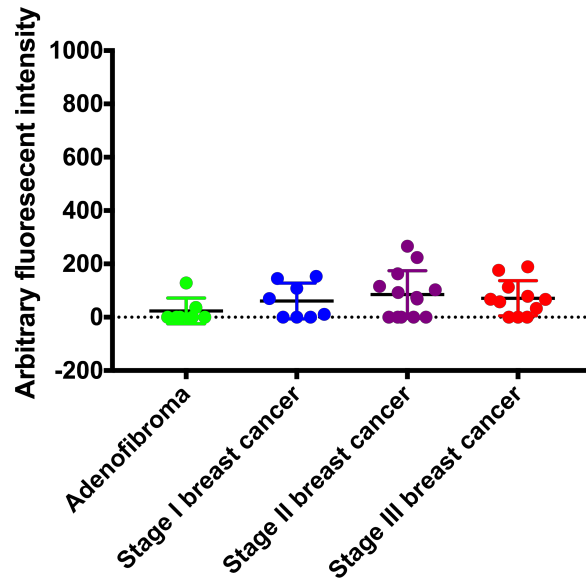


Figure 4-9: Expression analysis of PD-1, KLRG-1 and Tim-3 on T-cells from breast fibroadenoma and different TNM stages breast cancer individuals. A, C, E are the inhibitory receptors expression on tissue infiltrated T-cells in (%); B, D, F are the ligands expression on FFPE samples from matched patients quantified by confocal microscopy Zeiss 880.

In conclusion, from the paired data between the expression level of inhibitory receptors and ligands in different subgroups of breast diseases individuals, we have observed that PD-1 expression are relatively low in the benign breast tissues (fibroadenoma) compared with its expression in malignant tissues although no differential expression of PD-1 on T cells was found between fibroadenoma and Stage I breast cancers. This suggests that tumor infiltrated T cells in the early stage of breast cancer may have less exhausted phenotype with low expression of PD-1, although further functional assays are essential to confirm this founding. Of note, in the comparison of PD-L1 expression level in the tissues between benign breast disease and breast cancers, PD-L1 expression in the fibroadenoma tissues is significantly lower than that in different stages of tumor microenvironments respectively, which indicates that in non-triple negative breast cancer individuals, PD-L1 does have the predictive value for diagnostics to distinguish benign tissues from malignant cancers. To our knowledge this is the first cohort study that provide expression level of PD-L1 in non-triple negative breast cancer cohort. It indicates that non-triple negative breast cancer individuals may also benefit from PD-1/PD-L1 inhibitory blockade immunotherapy to induce anti-cancer T cells responses and may reduce the potential metastasis. In addition, these results may also further suggest the possibilities for the dual blocking of PD-1/PD-L1 axis on both inhibitory receptor (PD-1) blockade and ligand (PD-L1) to sustain and enhance anti-tumor T cell responses.

In the analysis of KLRG-1/e-cadherin and Tim-3/Galectin-9 expression on same cohort, no differential expression was detected within 4 different subgroups of patients. However, although limited number of patients is available in each subgroup, we still found KLRG-1 expression on CD8 TILs in stage I breast cancer has 2 distinctive expression clusters. Indeed, 4 out of 8 (50%) patients had more than 20% of KLRG-1 expression on CD8 TILs, whereas the remaining 4 out of 8 (50%) patients had less than 10% of KLRG-1 expression on CD8 TILs. Clear separate distribution of dot plots can be observed. However, this trend has not been observed in e-cadherin, one of its ligands, expression in tumor microenvironment from matched patients. KLRG-1 expression in stage II breast cancer has the highest mean expression on both CD4 and CD8 among all 4 subgroups.

The same expression pattern was also found in its ligand expression in tissues. KLRG-1 has been known as inhibitory receptor and T-cell differentiation marker[128]. Its expression may also link with aging[249]. Our results indicate that further investigation of KLRG-1 and its ligands should take the multiple functions KLRG-1 has into consideration. In addition, KLRG-1 enables to bind with 3 different ligands with different affinities inducing inhibitory signalling[132]. This may indicate that the expression of additional ligands, such as n-cadherin and v-cadherin that may also interact with KLRG-1, which should be simultaneously investigated in breast tumor microenvironment.

Tim-3/ galectin-9 inhibitory pathway is one of the most promising targets in immunotherapy apart from the approval drug by FDA in CTLA-4 and PD-1 blockades[171, 215, 216]. Co-expression of Tim-3 and PD-1 on virus specific T cells associate with virus persistence in chronic HCV infection[211]. Our data suggested that both Tim-3 expression on T cells and Galectin-9 expression in breast fibroadenoma and breast cancers are low overall when compared with the expression level of PD-1.

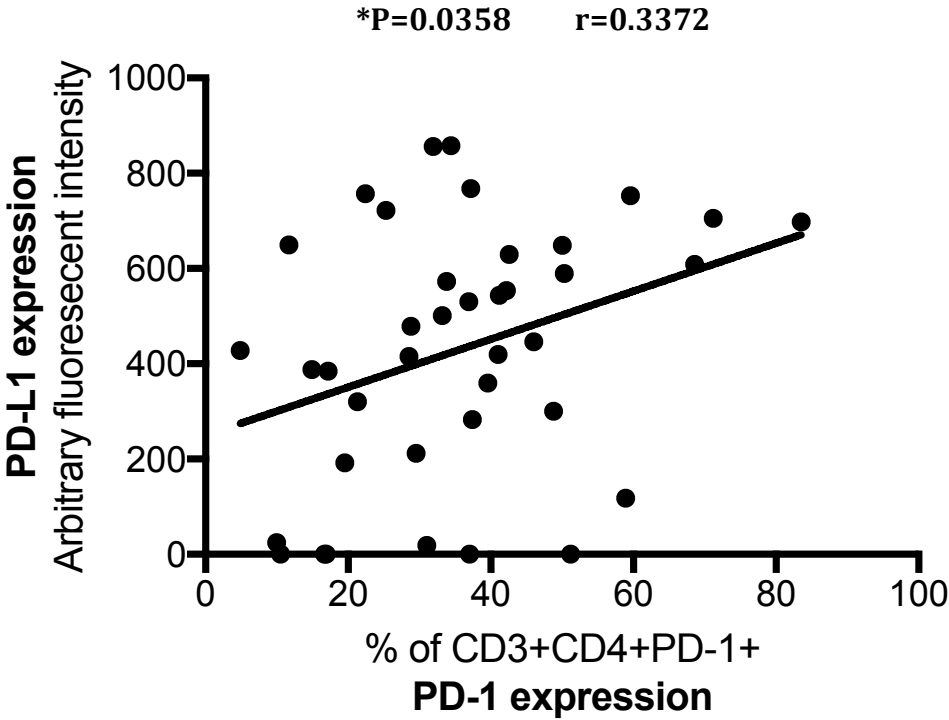
Considering the results from Chapter 3, that Tim-3 expression was significantly up-regulated on T cells in malignant tissues but not benign breast lesions when compared with its expression level on T cells in the peripheral blood respectively, Tim-3 may be an alternative target to block for the restoration of exhausted T cells in tumors. In addition, no differential expression of both Tim-3 and Galectin-9 was observed between benign breast tissues and different subgroups of malignant tissues. This suggests that in breast cancer the expression of Tim-3 and its ligand may have physiological roles. Hence, concerns should be taken during the evaluation of the application of Tim-3 inhibitors in immunotherapies, although Tim-3 is preferentially expressed in normal colorectal tissues but not breast tissues[107]. Therefore, we are still positive about the blockade of Tim-3/galectin-9 pathway in advanced breast cancer treatment, as it may be an alternative target to resuscitate dysfunctional T cells and elicit sustainable anti-tumor responses.

4.2.4 Correlation between PD-1/PD-L1 was observed in paired breast cancer

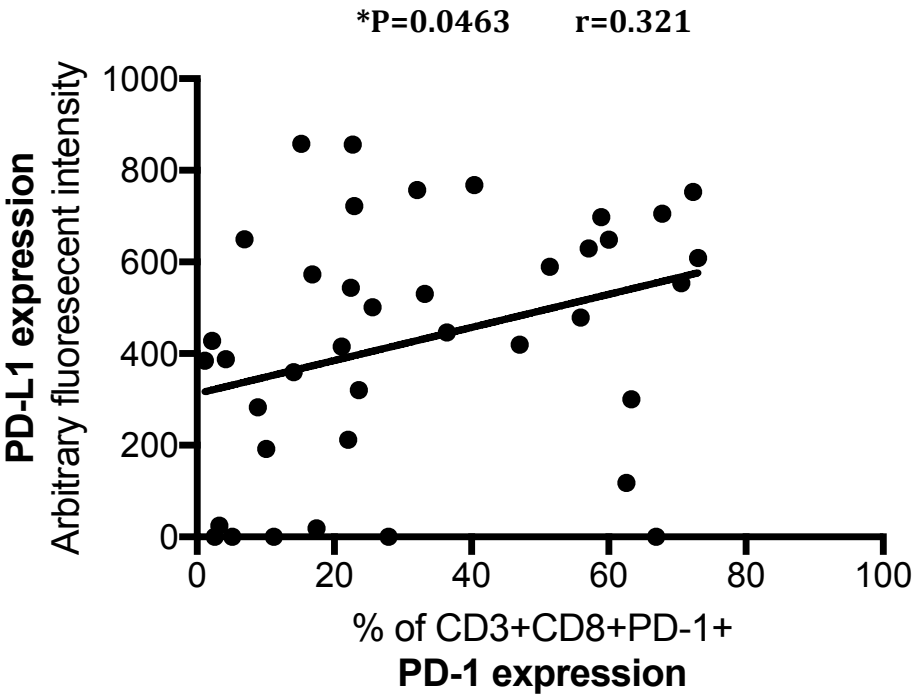
Previous clinical trials of PD-1/PD-L1 axis blockade immunotherapy indicate pivotal information in PD-L1 expression in differential tumor microenvironment[245, 250, 251]. Further, PD-L1 expression has been proposed to be the predictive biomarker in blockade to predict the outcomes of PD-1 blockade treatment, but the correlation of PD-L1 expression level and response rate of PD-1 inhibitors varies in different cancers[69]. For instance, results published from more large cohort studies, in Phase III randomized clinical trial of melanoma (n=411), 13% of PD-L1 negative patients also benefited from Pembrolizumab treatments[78]. Similar clinical trial results were found in Nivolumab treatments that 17% of PD-L1 negative patients had responses after treatments[48]. It indicates in different cancers, even in different subtypes of the same cancer, PD-L1's predictive value should be investigated independently. Therefore, in our breast cancer cohorts, we analyzed if there is correlation between inhibitory receptor expression on T cells and its ligand expression in tumor microenvironments. This correlation may indirectly provide the predictive value of PD-L1 as a biomarker in treating breast cancer individuals by Nivolumab or Pembrolizumab. In parallel, we have also conducted the correlation test of KLRG-1/e-cadherin and Tim-3/galectin-9 in matched 39 breast cancer individuals. This may further demonstrate that which inhibitory pathway could be a predictive marker and which pathway could be the most promising target to block in breast cancer individuals

Figure 4-10: Correlation between inhibitory receptor expression on TILs and ligands expression in tumor from breast cancer individuals

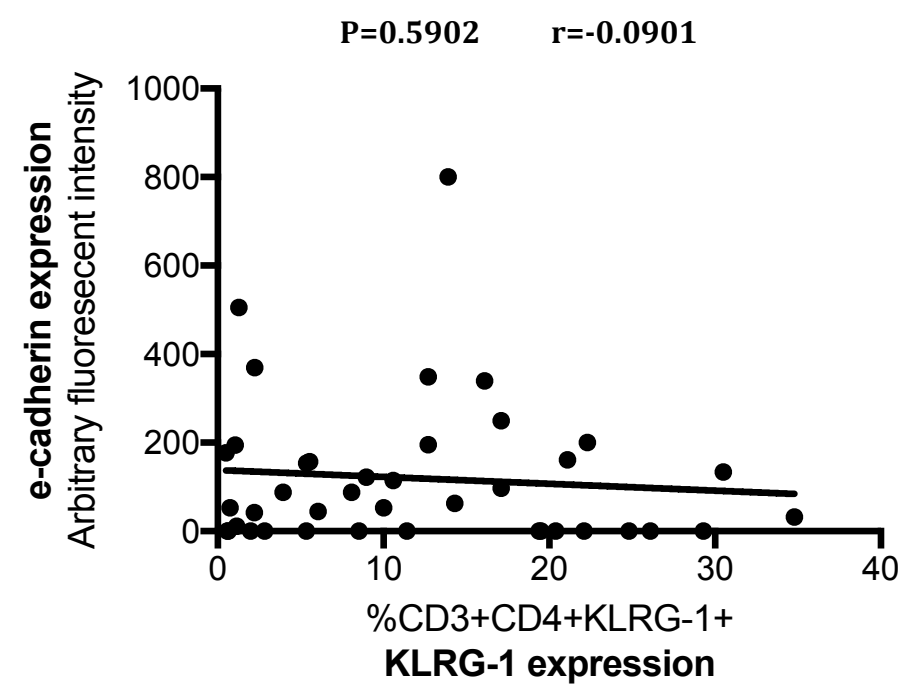
A: Correlation between PD-1 expression on CD4 TILs and PD-L1 expression in breast cancer



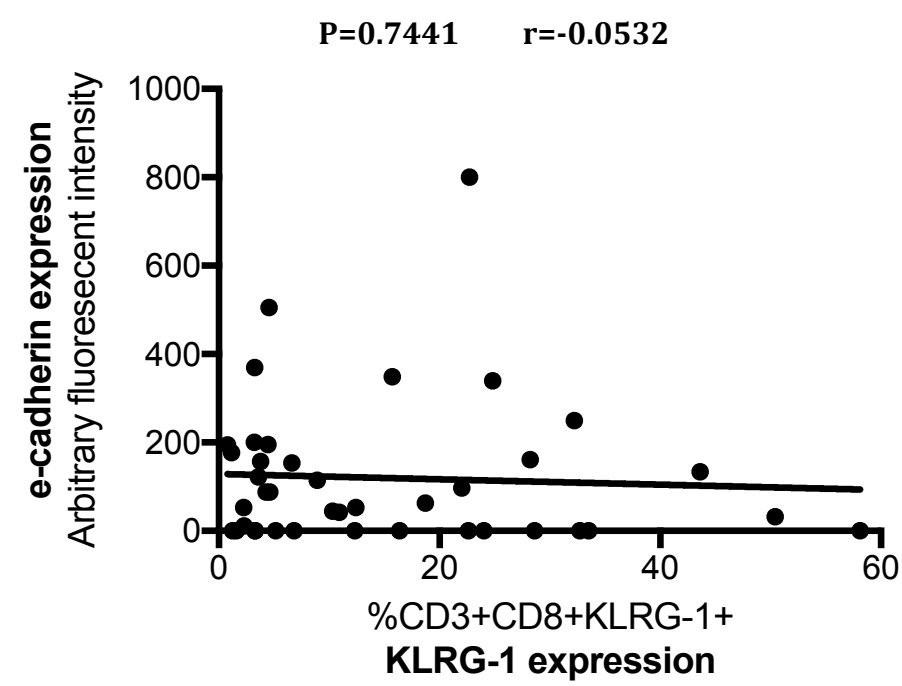
B: Correlation between PD-1 expression on CD8 TILs and PD-L1 expression in breast cancer



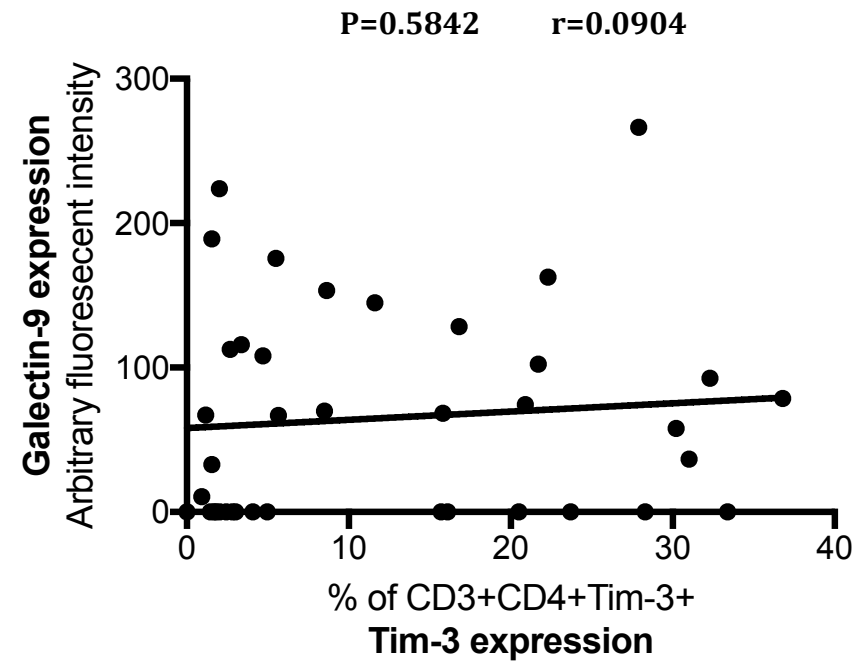
C: Correlation between KLRG-1 expression on CD4 TILs and e-cadherin expression in breast cancer



D: Correlation between KLRG-1 expression on CD8 TILs and e-cadherin expression in breast cancer



E: Correlation between Tim-3 expression on CD4 TILs and galectin-9 expression in breast cancer



F: Correlation between Tim-3 expression on CD4 TILs and galectin-9 expression in breast cancer

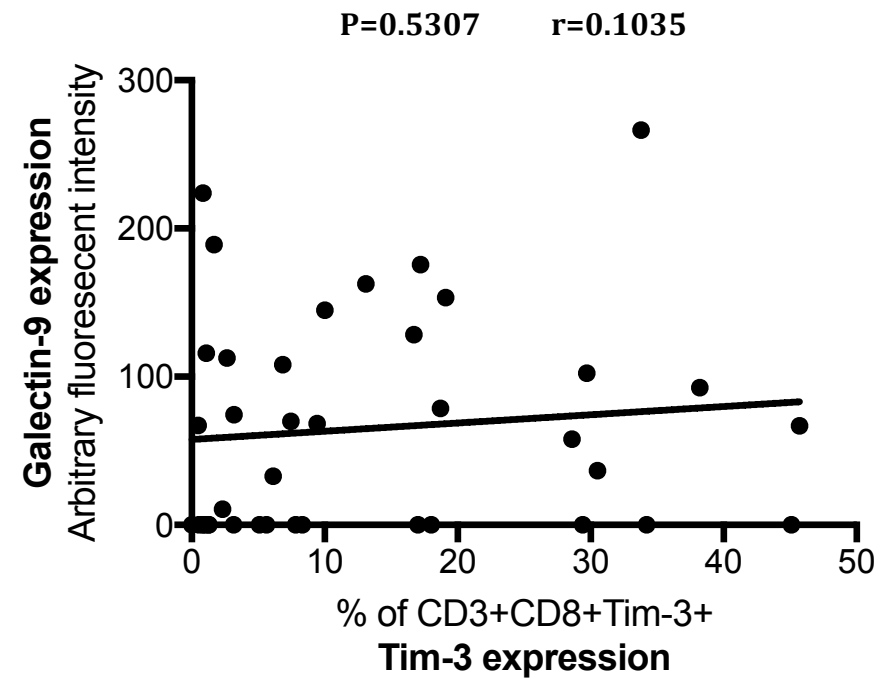


Figure 4-10: A: correlation test between PD-1 expression on CD4 T cells and PD-L1 expression in breast cancer, PD-1 expression on CD4 TILs has a moderately positive correlation with PD-L2 expression in breast cancer; B: correlation test between PD-1 expression on CD8 T cells and PD-L1 expression in breast cancer, PD-1 expression on CD8 TILs has a moderately positive correlation with PD-L2 expression in breast cancer; C and D: no positive or negative correlation detected either between KLRG-1 expression on CD4 TILs and e-cadherin expression in tumor or KLRG-1 expression on CD8 TILs and e-cadherin expression in tumor; E and F: no positive or negative correlation detected either between Tim-3 expression on CD4 TILs and galectin-9 expression in tumor or between Tim-3 expression on CD8 TILs and galectin-9 expression in tumor; Pearson correlation coefficients test.

In conclusion, when effector cells in tumor microenvironment release interferon-gamma, it up-regulates the expression of PD-L1 on tumor cells and antigen presenting cells, which then interact with PD-1 on T cells and further inhibit CTL function of TILs through PD-1 signaling. As a result, T-cells adoptively maintained an exhausted phenotype in the tumor microenvironment as characterized by up-regulation of PD-1 and other inhibitory receptors[70]. Our data indicates that in 39 paired breast cancer cohorts, PD-1 expression on either CD4 or CD8 tumor infiltrating T cells, positively correlate with the expression of PD-L1 in tumor microenvironment. This may indicate the PD-L1 may have the value as a biomarker in the treatment of non-triple negative breast cancer individuals by PD-1 pathway blockade. Quantifying PD-L1 expression by fluorescent intensity through confocal microscopy allow us to measure the magnitude of expression. However we still could not answer another question being, which expression level of PD-L1 in breast cancer tissues may indicate overall responses when administering PD-1 inhibitors. With more clinical trials in the application of Nivolumab and Pembrolizumab on multiple cancers, we may have more clues. Many factors may contribute to the dynamic balance between PD-1 expression on T cells and PD-L1 expression in tumor. Currently, PD-1

inhibitor clinical trials have been applied to TNBC (triple negative breast cancer)-ER negative, PE negative and HER-2 negative, a poorest prognosis subtype within breast cancers. In our cohort, only one individual recruited is TNBC, which rules out the confounding factors when conducting the analysis since TNBC may have distinctive carcinogenesis different from other subtypes of breast cancers, differentially harvesting immunosuppressive tumor microenvironment. In non-TNBC individuals, 5-year survival rate of stage I breast cancer is over 90%[252-255]. But the 5-year survival rate dropped to 60% in stage III breast cancer individuals[253-255]. Hence the PD-1/PD-L1 inhibitory immunotherapy may apply to late stage non-TNBC individuals to prolong its survival and preventing recurrence.

Our analysis observed neither positive nor negative correlation between KLRG-1/e-cadherin and between Tim-3/galctin-9 inhibitory pathways. This may result from multiple ligands that could interact with KLRG-1 and Tim-3 respectively. KLRG-1 could bind to 3 different classical cadherin family ligands; e-cadherin, v-cadherin and n-cadherin[132]. In vitro studies have indicated that all 3 ligands can bind to KLRG-1 on T cells and NK cells for the inhibition of the killing function, to cadherin expressed cells. Our results suggested that the expression of KLRG-1 on TILs is independent of e-cadherin expression in breast tumors. But it is uncertain that if KLRG-expression correlates with another 2 cadherin ligands, n-cadherin and v-cadherin. To further analyze its inhibitory function, 3 ligands expression and its affinity with KLRG-1 as well as its contribution to inhibitory signalling should be performed.

4.3 Discussion

Since the approval of the first immune checkpoint blockade, ipilimumab, in 2012 by FDA, numerous advanced cancer patients benefited from blockade immunotherapies. For instance, PD-1 inhibitors received 30-40% overall response rate in treating advanced melanoma, 19% ORR in treating non-small cell lung cancer and 20%- 25% ORR in treating renal cancers[52, 72, 78, 239, 240, 245, 256]. However, neither PD-1 inhibitors received positive responses in all types of solid tumors nor all the patients could benefit from PD-1 blockade in the "responsive" type of cancer[257]. This drives us to next critical question that which biomarker could distinguish responders and non-responders before the blockade therapy. PD-L1 has been proposed to be the one of the most promising biomarker to predict who may demonstrate responses in PD-1/PD-L1 pathway blockade[48, 72, 250, 251].

However, with accumulated results revealed from multiple clinical trials, it is still controversial to define PD-L1 as a biomarker as in PD-L1 negative tumors, 17%-19% of advanced melanoma patients also had responses to Nivolumab[240]. This involves several considerations. First, tumor microenvironment is complex, which consists multiple factors contributing to immunosuppressiveness and dysfunction of TILs. Additional inhibitory pathways other than PD-1/PD-L1 may be the dominant checkpoints to induce T-cell exhaustion. Secondly, the biopsy of tumor tissues for the quantification of PD-L1 may bring biases as different locations of solid tumor may harvest differential expression of PD-L1. This is particularly important in defining PD-L1 positivity in large solid tumors as the center of tumor is highly hypoxia and therefore may

alter the expression of PD-L1 or other potential biomarkers. Thirdly, current quantification of PD-L1 in multiple clinical trials is conducted by automated IHC in which the cut-off value and the choice of PD-L1 antibodies may generate different interpretations.

Hence, correlation between PD-1 expression on TILs and PD-L1 expression in tumor microenvironment in a subtype of cancer is the prerequisite to evaluate whether PD-L1 could be the potential biomarker in the specific type of cancer. In our studies, we used fluorescent intensity in confocal microscopy, a more sensitive and stable quantification method, to quantify the PD-L1 expression in breast cancer tumor microenvironments. Indeed, we have found the moderate correlation between the expression PD-1 on TILs and the expression of PD-L1 in TME from non-triple negative breast cancer individuals. Although we did not recruit advanced non-triple breast cancer individuals from PD-1 inhibitors clinical trial, it still indicated that advanced non-triple negative breast cancer individuals may benefit from PD-1 inhibitor as well.

Confocal microscopy is superior in quantitative analysis but different from IHC, the positivity of PD-L1 should be defined in another method. Since, we have observed quite low expression of PD-L1 in breast fibroadenoma individuals, with the enlarged sample size, we may enable to define a cut-off value of PD-L1 positivity. In addition, we did not observe correlation either from KLRG-1 and e-cadherin or from Tim-3 and Galectin-9. Since both inhibitory receptors have multiple ligands with different binding affinities, it is important to quantify the

expression of those ligands with the expression of inhibitory receptors simultaneously, which would be our future work.

In all, our interpretation is that PD-L1 may be a biomarker in guiding the application of the blockade immunotherapies to patients who may demonstrate responses to. But its value as a biomarker should be evaluated in different subtypes of cancers respectively.

5 Chapter 5: characterizing T-cell exhaustion in HBV chronically infected liver diseases

5.1 Introduction

Within a few years to few decades, chronic HBV infection progresses to HBV associated liver cirrhosis commonly with liver damage, clinically presenting jaundice, ascites and dysfunction of protein synthesis. Inside the liver, this particular stage of disease may have 2 different outcomes; 1: it induces malignant transformation-establishment of HCC; 2: decompensated liver cirrhosis without appropriate liver donors for liver transportation finally terminates patients life.

In this chapter, we aim to characterize the T-cell exhaustion by the expression of inhibitory receptors in HBV chronically infected individuals from cross-sectional chronic HBV cohort, in which peripheral blood was collected from chronic hepatitis B individuals, HBV associated liver cirrhosis individuals and HBV associated HCC individuals. Furthermore, we have also applied the bioinformatics approaches to identify the heterogeneity of peripheral lymphocytic subsets at single-cell level. Multiple algorithmic models including CITRUS SPADE and viSNE were used to predict and reveal the significant immune-signatures within different HBV associated disease stages. In addition, for those individuals who demonstrate distinctive phenotypic characteristics, such as higher expression of one or several inhibitory receptors on T cells when comparing with that of the rest of patients in the same group, viSNE was applied to elucidate the immune-signatures of that specific individual at single-cell level

in 2 dimensional plots while maintaining the distance between cells.

5.2 Results

5.2.1 No differential expression of inhibitory receptors on peripheral T cells were observed among different stages of HBV chronically infected individuals

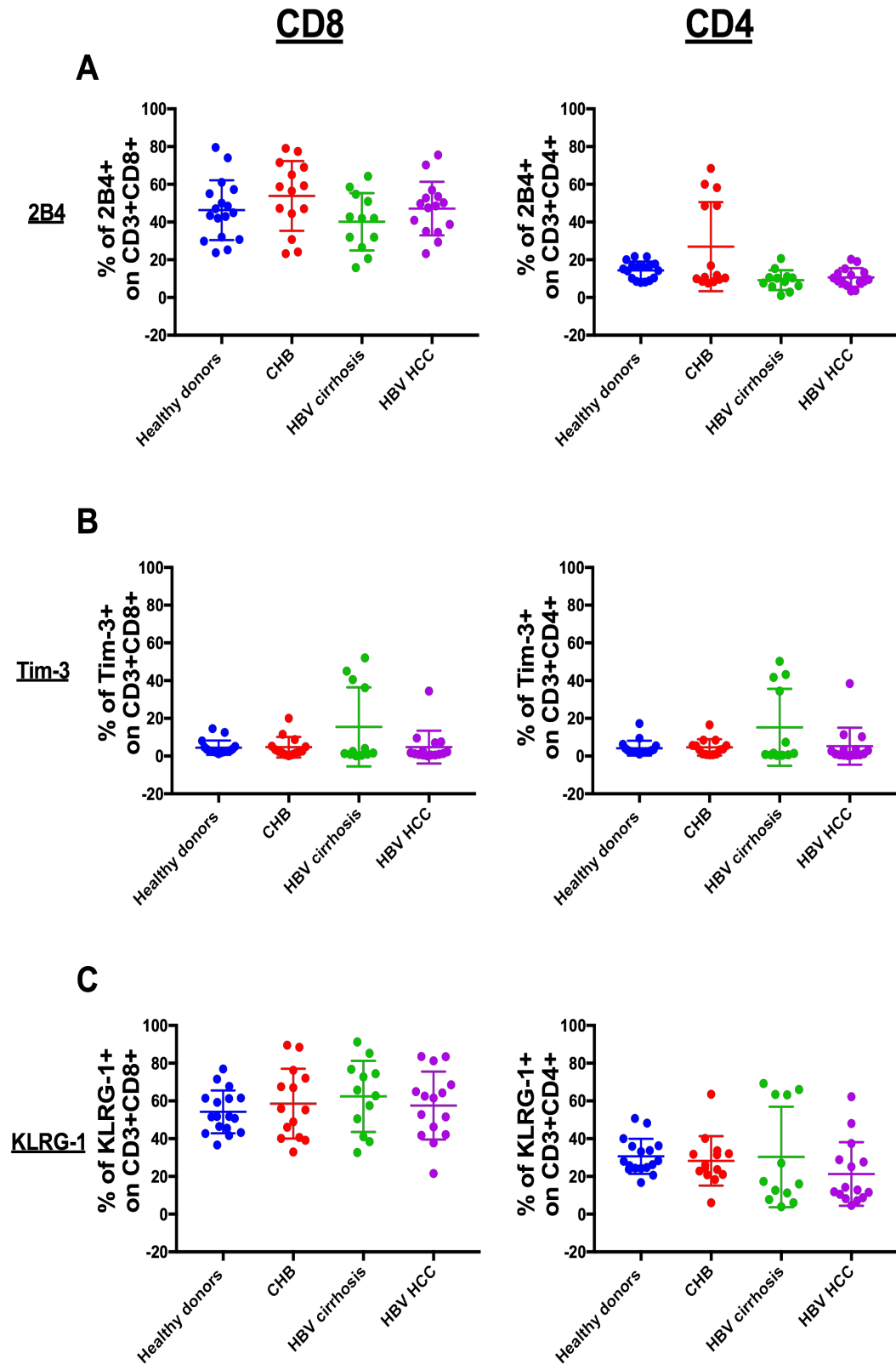
Chronic HBV infection dampens HBV specific T cells function with impaired cytokine production and the up-regulation of multiple inhibitory receptors, such as PD-1, CTLA-4 and 2B4 defined as T-cell exhaustion[202]. Both CD4+ T cells and CD8+ T cells involve in the immune responses against HBV through distinctive pathways[258]. Therefore, characterizing the expression of inhibitory receptors on both CD4+ T cells and CD8+ T cells in different stages of HBV chronically infected individuals may provide crucial information for the reinvigoration of exhausted T cells under chronic HBV infection.

In our HBV chronic infection cohort, we have in total recruited 66 individuals ranging from chronic HBV hepatitis (14/66), HBV associated cirrhosis (11/66), HBV associated HCC (15/66) and healthy donors (16/66). Clinical information is summarized in Table 5-1. 14-color multichromatic panel (panel detail attached in Chapter 2) was designed to characterize the expression of 6 inhibitory receptors including PD-1, CTLA-4, Tim-3, 2B4, KLRG-1 and TIGIT on T cells from peripheral blood of individuals in our cohort..This set of experiments intends to identify the potential significance in inhibitory receptor expression on circulating T cells from individuals in different disease stages.

Table 5-1:

Total samples:		66
Gender:		Male 55% (36/66) Female 45% (30/66)
Age:		52.39±14.29 years
Classification:	Healthy donors	24% (16/66)
	CHB	21% (14/66)
	HBV cirrhosis	17% (11/66)
	HBV HCC	23% (15/66)

Figure 5-1: Expression analysis of inhibitory receptors on both CD4+ and CD8+ T cells among healthy donors, CHB, HBV associated cirrhosis and HBV associated HCC individuals



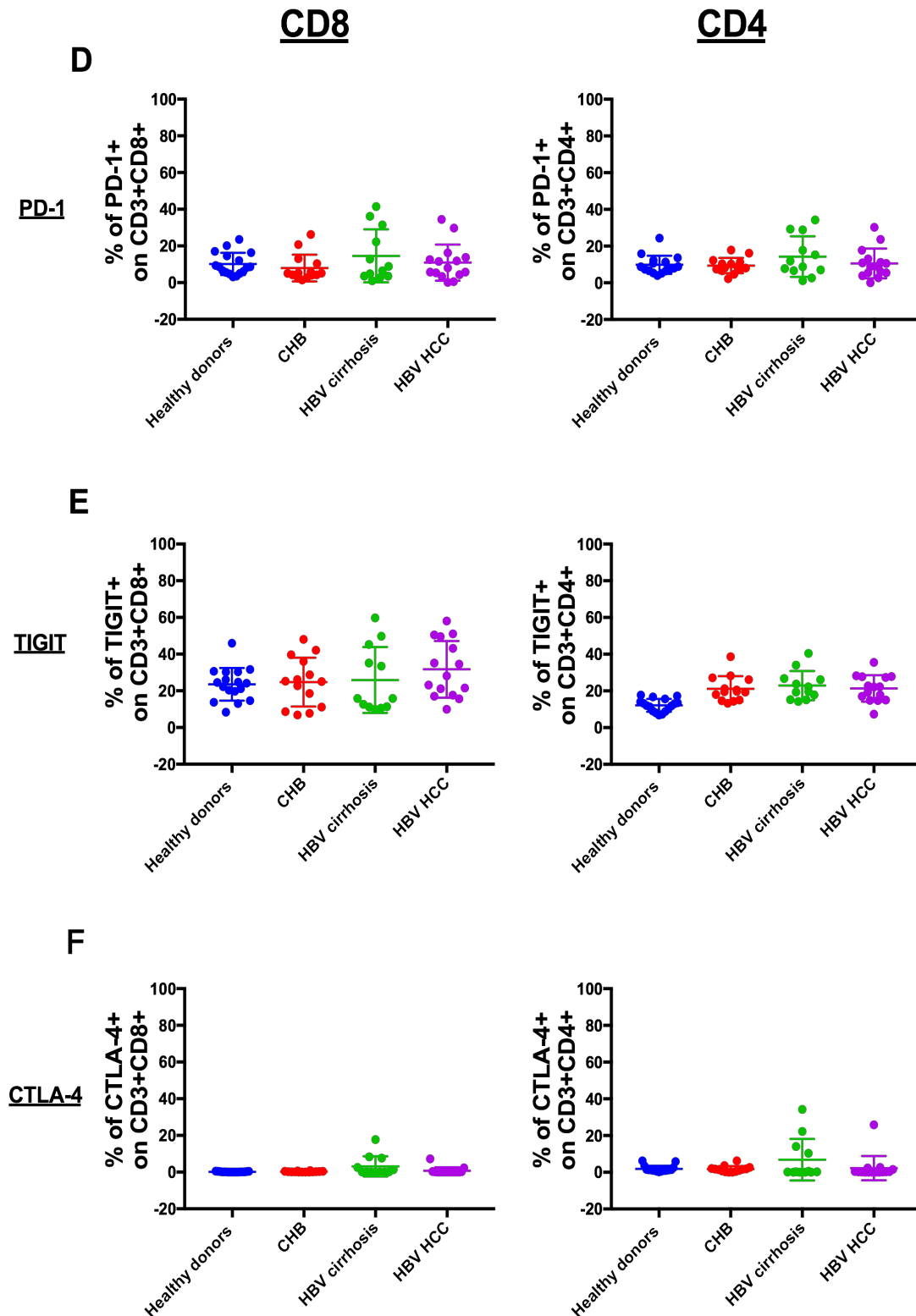


Figure 5-1: Expression analysis of PD-1, TIM-3, 2B4, KLRG-1, TIGIT and CTLA-4 on CD8+ (two left graphs) and CD4+ T-cells (two right graphs) on PBMCs from 16 healthy donors, 14 CHB individuals, 11 HBV associated cirrhosis individuals, 15 HBV associated HCC individuals. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney t-test). **A)** 2B4, **B)** TIM-3, **C)** KLRG-1, **D)** PD-1, **E)** TIGIT, **F)** CTLA-4, expression on CD4+ and CD8+ T cells on PBMCs in (%).

Having hypothesized that there is differential expression of inhibitory receptors on both CD4⁺ T cells and CD8⁺ T cells among different stages of HBV chronically infected individuals, we were surprised to obtain the results demonstrated in Figure 5-1 that no differential expression of PD-1, Tim-3, 2B4, KLRG-1, TIGIT and CTLA-4 on circulating T cells was observed within 4 subgroups of individuals. Unexpectedly, there was no differential expression of those inhibitory receptors on T cells between healthy donors and any HBV disease subgroups.

However, from Figure 5-1-A, 2 well-separated sub-clusters in 2B4 expression on CD4 in CHB individuals should be noted. Specifically 5 out of 14 individuals in CHB subgroup have over 40% of 2B4 expression on CD4 T cells. Meanwhile, the rest 9 out of 14 individuals in this subgroup, only present 20% of 2B4 expression on CD4 T cells on average. Similarly, in Figure 5-1-B, 2 well-separated sub-clusters in Tim-3 expression on both CD4 and CD8 T cells in HBV associated liver cirrhosis subgroup was observed. 4 out of 11 HBV associated cirrhosis individuals exhibited above 30% of Tim-3 expression on both CD4 and CD8 T cells whereas the other 7 individuals in the same cohort were below 20%. Linking the clinical information, it was found that 4 individuals with high Tim-3 expression on CD4 T cells and 4 individuals with high Tim-3 on CD8 T cells are identical patients.

Generally, our interpretation is that all the HBV chronically infected patients in our cohort were on the antiviral treatment (ART) regimen in which the HBV

virus load has been substantially controlled. In return, virus pressure may generate less influence to circulating T cells in the context of the expression of inhibitory receptors. In addition, circulating T-cells is a highly heterogeneous population that may mask the subpopulations exhibiting distinctive expression of inhibitory receptors among different disease stages. Therefore, the further investigation of T cells in the periphery from different stages of HBV chronically infected individuals should dissect the heterogeneity to reveal the immunological characteristics of exhausted T cells which may facilitate the development of new therapeutic approaches to resuscitate those dysfunctional T cells for potent responses against HBV virus and HBV virus associated cancer.

These findings also suggest that under similar disease stage, different patients could have a distinctive immunological status as indicated by differential expression of multiple functional markers and may correlate with the disease outcomes and drug responsiveness. In addition, the Tim-3 high expression subgroup of HBV associated cirrhosis individuals may have a high response rate when administering Tim-3 inhibitors. In all, these result also suggested that in the researches of identifying specific marker expression significance on T cells among different disease courses as predictive biomarkers or drug targets, it is probably advisable to dissect the heterogeneity of T cell population into single-cell level.

5.2.2 Applying bioinformatics analysis-CITRUS to reveal the heterogeneity of lymphocytes among different stages of HBV chronically infected individuals

Investigation and examination of lymphocytic subsets that may exert a prominent contribution to disease progression or recovery, is a common method in immunology research. Indeed, current technology of flow cytometry with an efficient fluidic system enables us to acquire numerous cells with multiple fluorescent-labeled antibody staining in short time. This allows us to analyze differential subsets simultaneously with limited cells per sample. In this thesis, we have designed a 14-color panel to investigate T-cell exhaustion in HBV chronic infection and cancer.

However, as the results shown in 5.2.1, conventional analysis of mono-marker expression on different T cell subsets could not address our research questions. In addition, data with high dimensionality generates a large number of possibilities in defining the phenotypes of subsets. For instance, our 14-color panel includes 6 inhibitory receptors and 7 markers for distinguishing subsets. Consequently, over 100 combinations of different lymphocytic subsets could be defined for the analysis. Hence the scalability and subjectivity in conventional manual-analysis of high dimensional FACS data could not address our hypothesis appropriately. Thereafter, CITRUS (Cluster Identification, Characterization, Regression), an algorithm based bioinformatics approach in stratifying subpopulations in high dimensional flow cytometric data was applied to analysing our abovementioned cohort.

CITRUS is an algorithm that leverages unsupervised clustering and supervised predictive or correlative statistical association models to identify significant features between experimental groups. In this study, we applied the Significance Analysis of Microarrays (SAM, correlative model) and Nearest Shrunken Centroid (PAMR, predictive model) models to investigate whether if any subsets are significantly changed in proportion or in expression of a marker, among different stages of HBV chronically infected individuals. The CITRUS rationale was illustrated in Figure 5-2 below.

Figure 5-2: Dividing heterogeneous population into hierarchical clusters

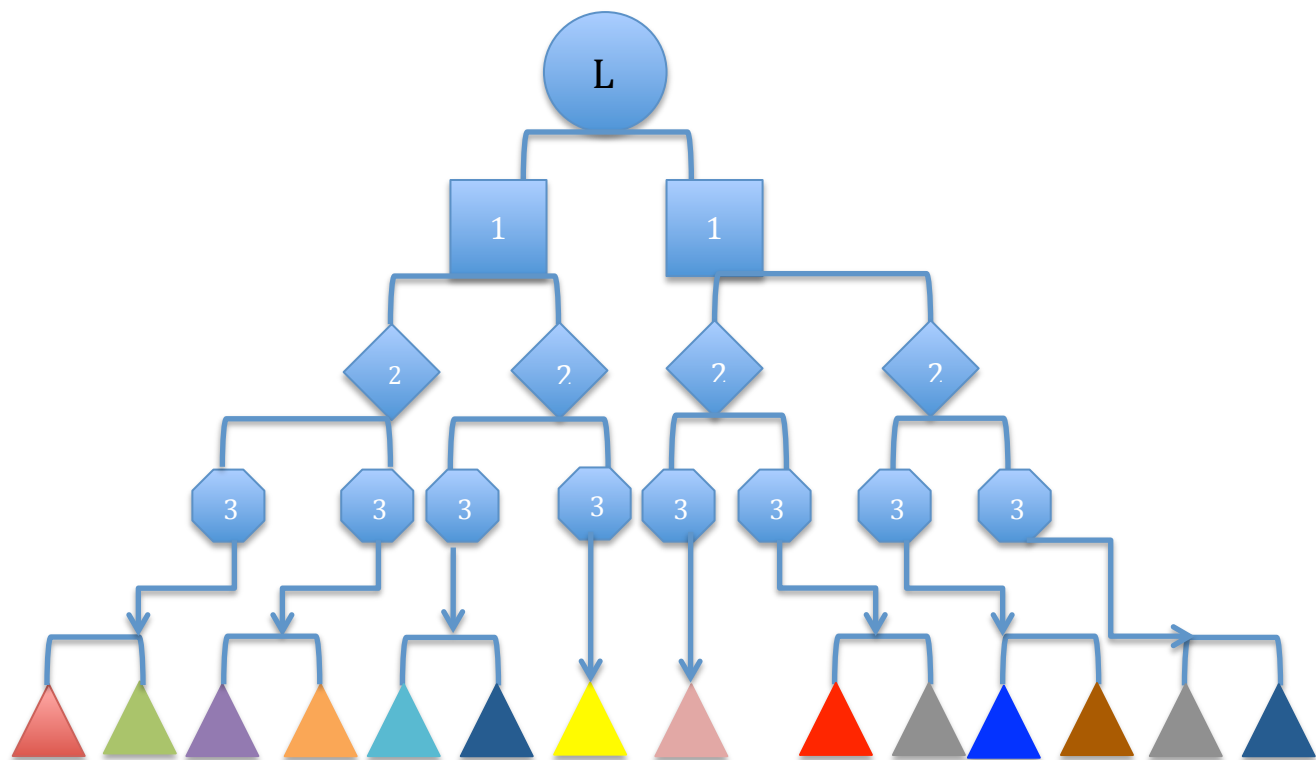


Figure 5-2: Cartoon above illustrates the rationale of CITRUS algorithmic model to divide lymphocytic population into cell clusters based on the expression of multiple markers in hierarchical manner. Top shape is entitled “L” representing whole population of lymphocytes. Shapes entitled “1” are the subsets sharing similar marker expression patterns containing inside “L”. Likewise, shapes containing “2” are the descendant clusters from shapes “1” and shapes “3” are divided from shapes “2”. But from shapes “1” to shapes “3”, they are all phenotypically similar. However, terminal shapes would exhibit distinctive phenotypes (in variable colors) that may have prominent roles.

5.2.2.1 CITRUS plots reveal the markers expression in differential lymphocytic subsets

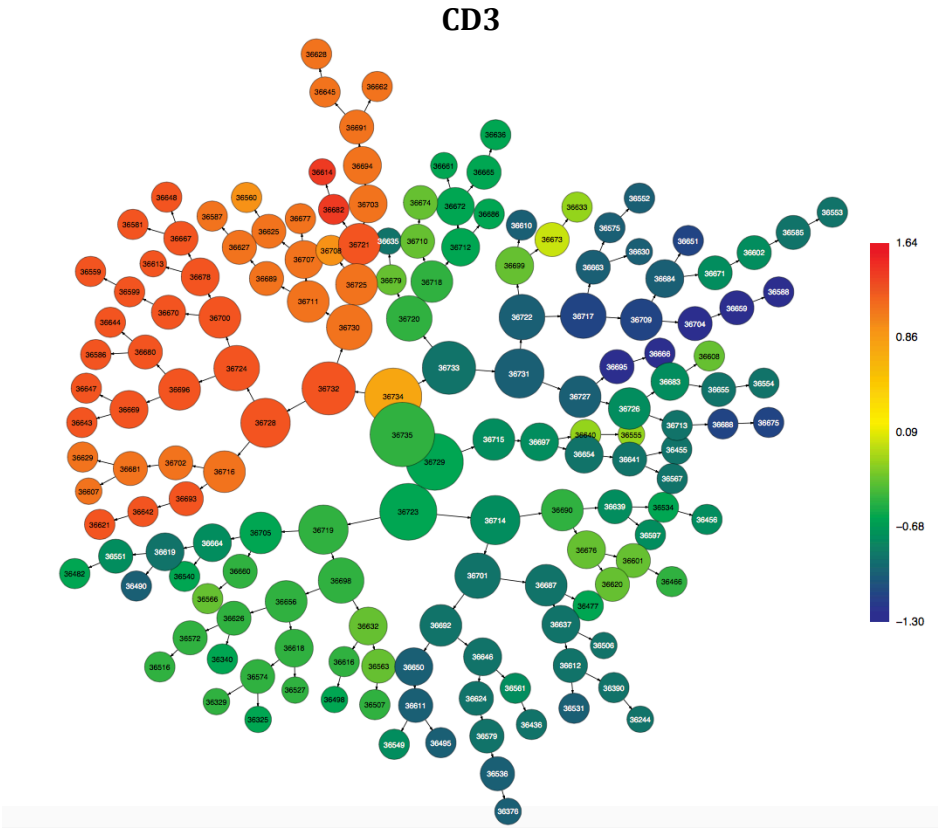
Flow cytometric data of 66 individuals including healthy donors, HBV chronically infected individuals, HBV associated cirrhosis individuals and HBV associated HCC individuals together with FMO control files were all uploaded into the Cytobank server

(https://premium.cytobank.org/cytobank/experiments/97470/advanced_analyses/citrus/). Groups entitled “Healthy donors”, “CHB”, “HBV cirrhosis” and “HBV HCC” had been defined and created based on the clinical stratification in the analysis before running the models.

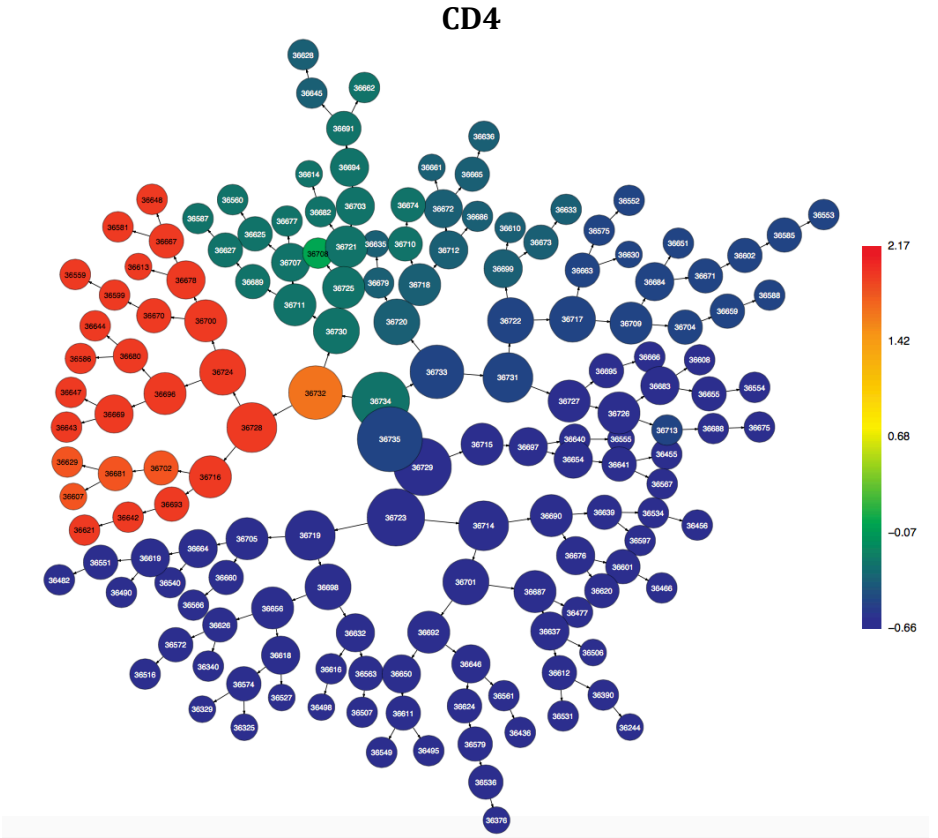
For plotting the CITRUS clusters, 656 events were sampled per file. They are 36736 events clustered in the CITRUS plot, with a minimum cluster size of 367 events, equally acquired from all the samples. Cross validation fold is 1 with 1% false discovery rate. As all the data was acquired by fluorescent flow cytometry rather than mass cytometry, data was running in CITRUS model in normalization scale mode in which each channel is normalized so that it has a mean value of zero and a standard deviation of 1. This is done by first concatenating all files within the CITRUS run, then calculating for each event value per channel, subtracting the mean and dividing by the standard deviation of the channel.

Figure 5-3: CITRUS plots based on 66 experimental subjects

A:

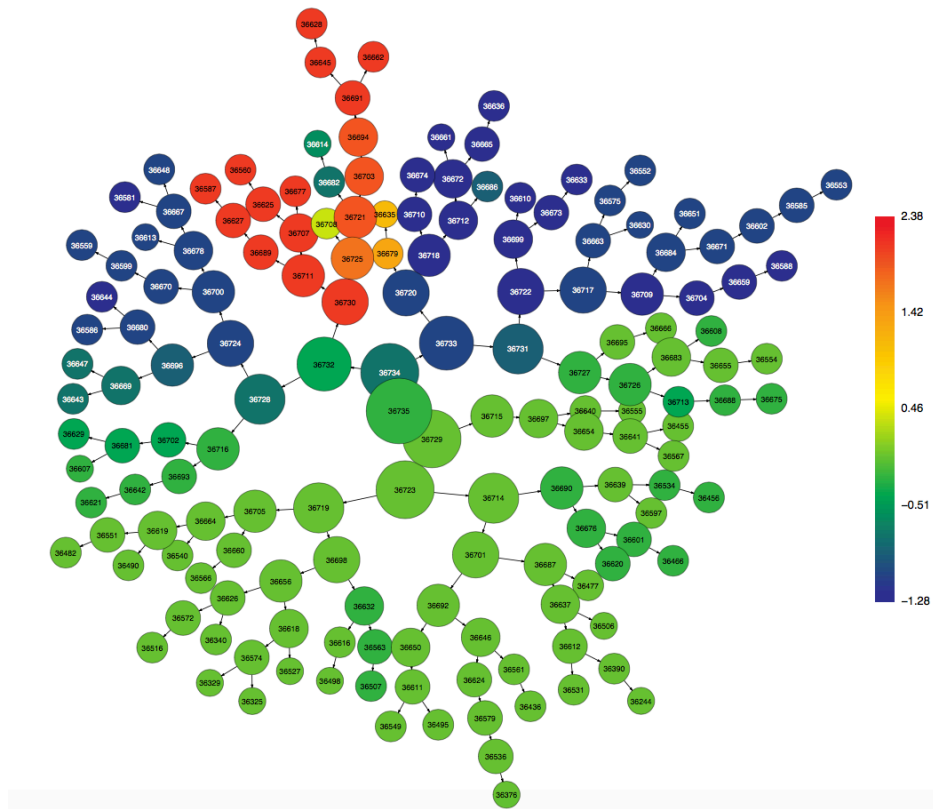


B:



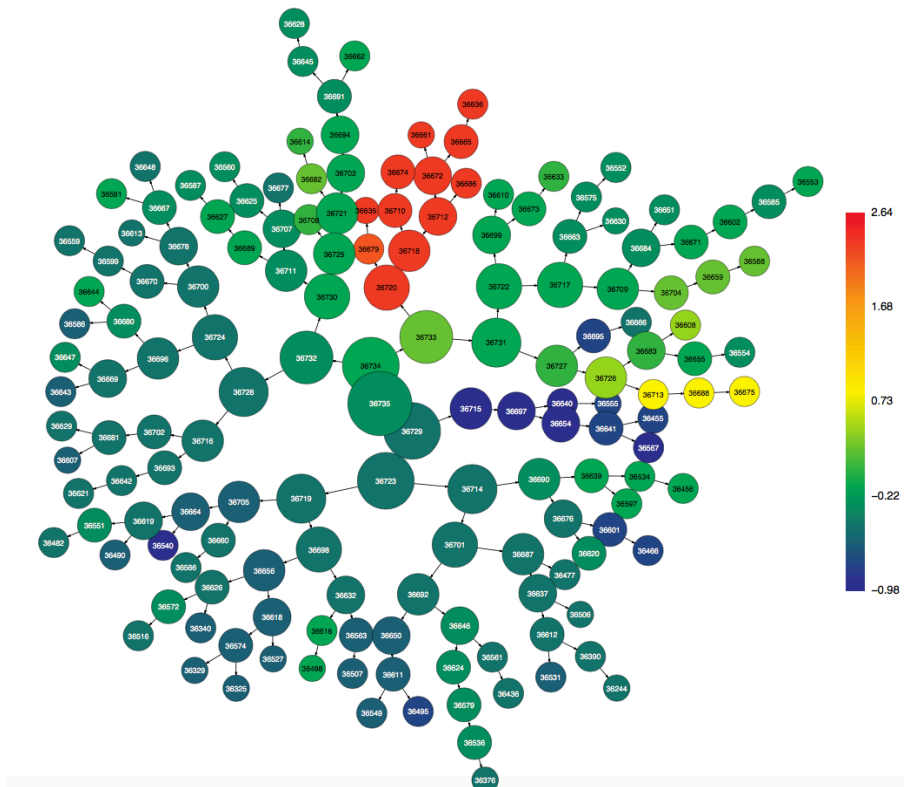
C:

CD8



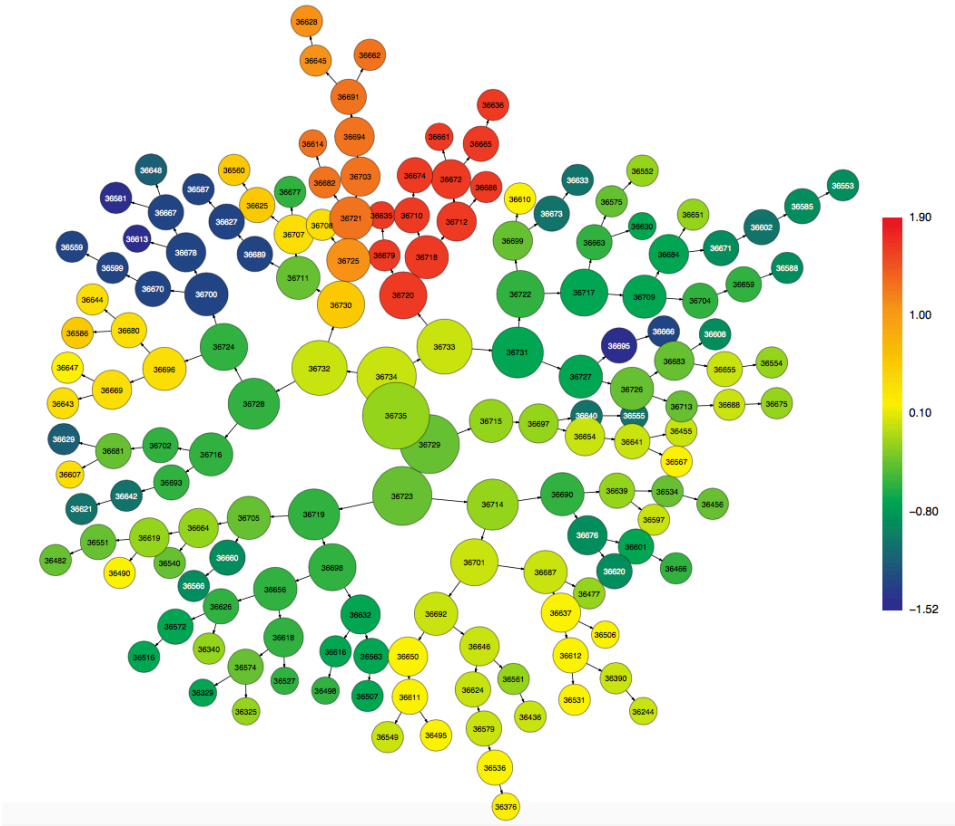
D:

CD56



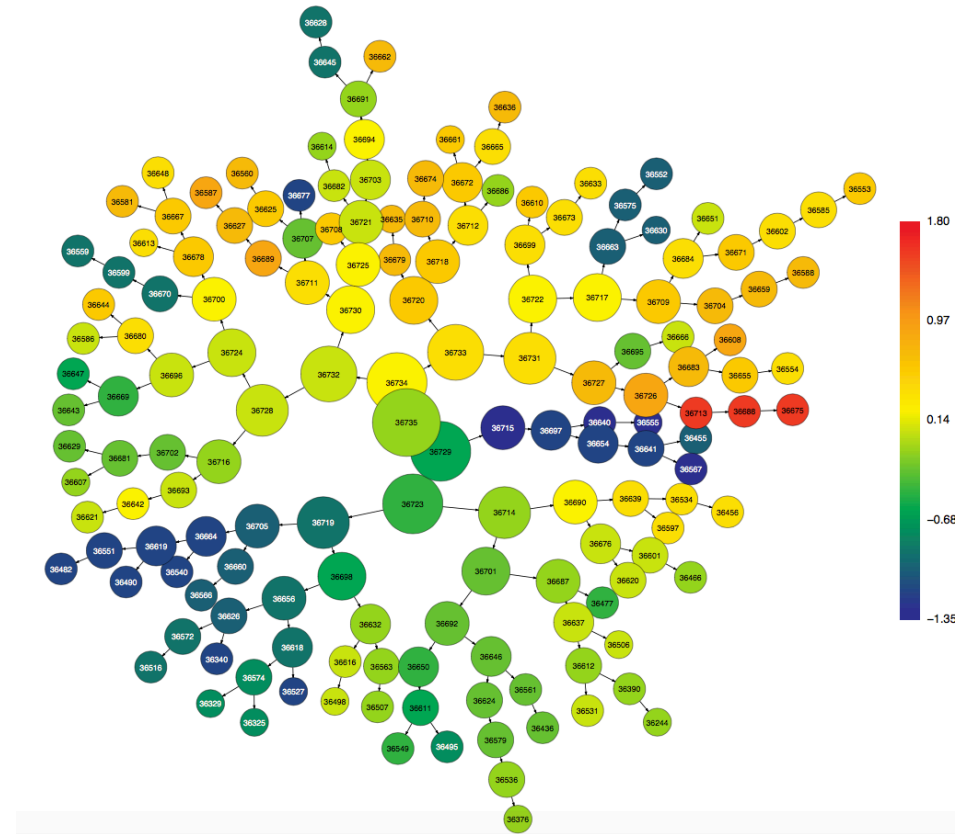
E:

2B4



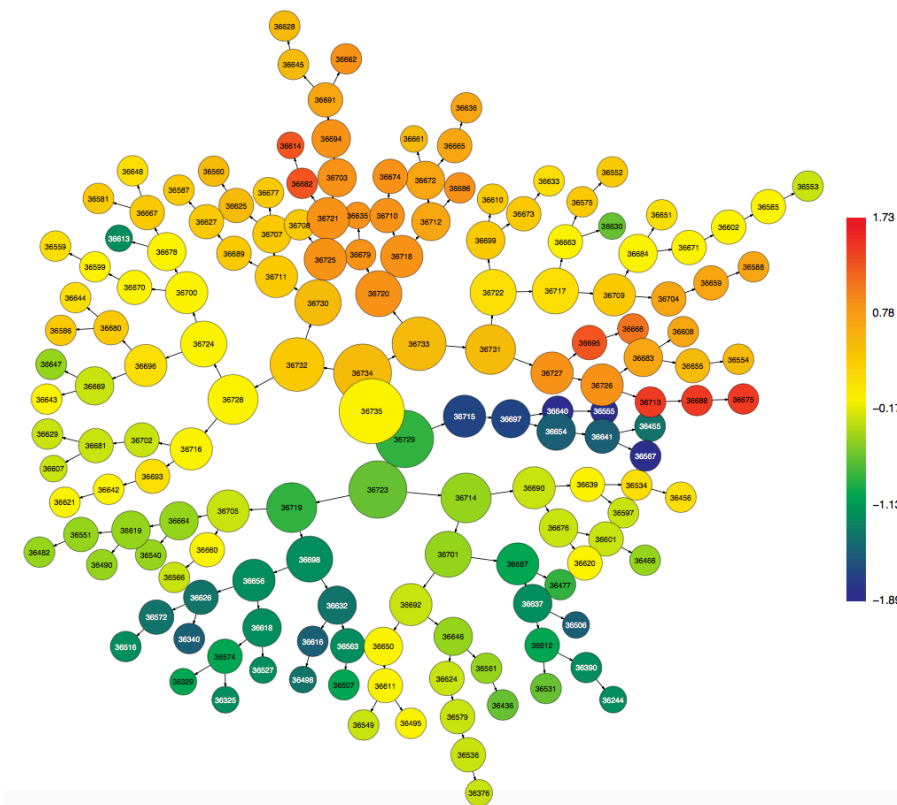
F:

CTLA-4



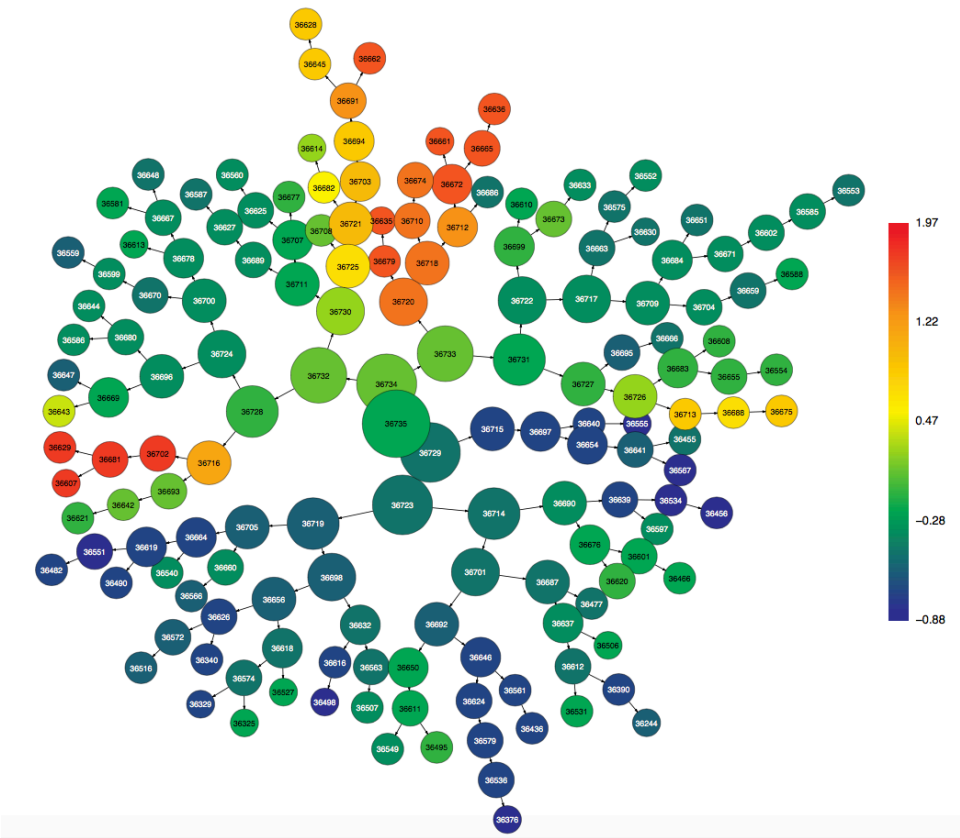
G:

KLRG-1



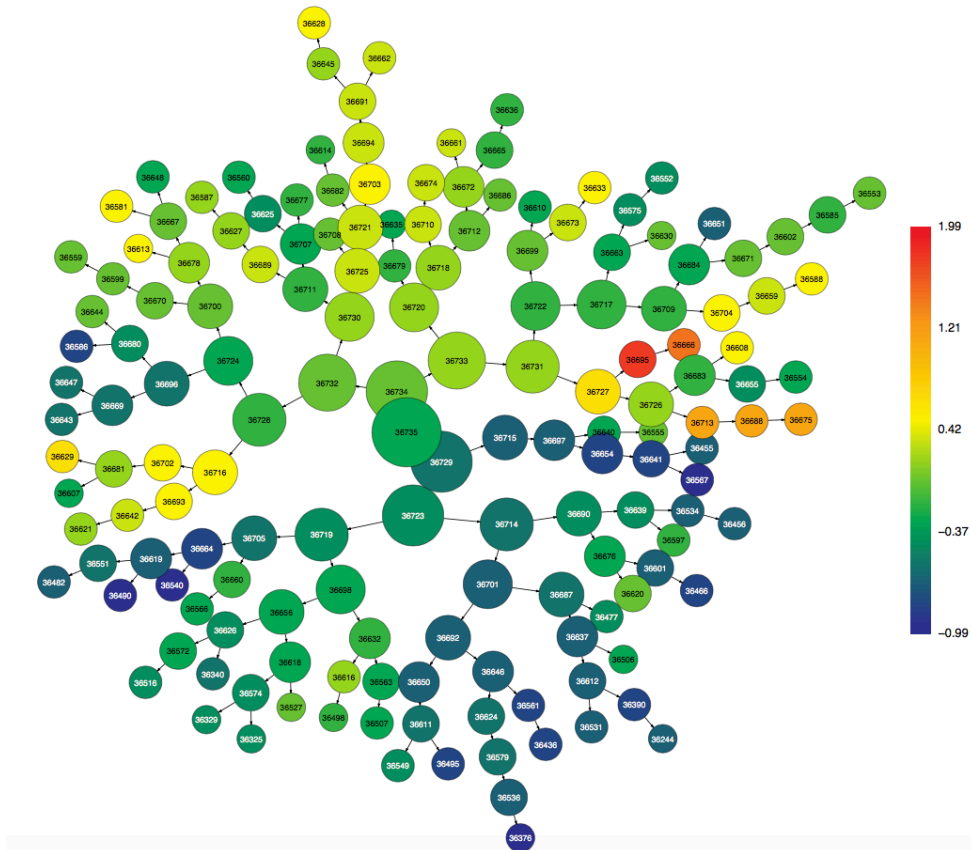
H:

TIGIT



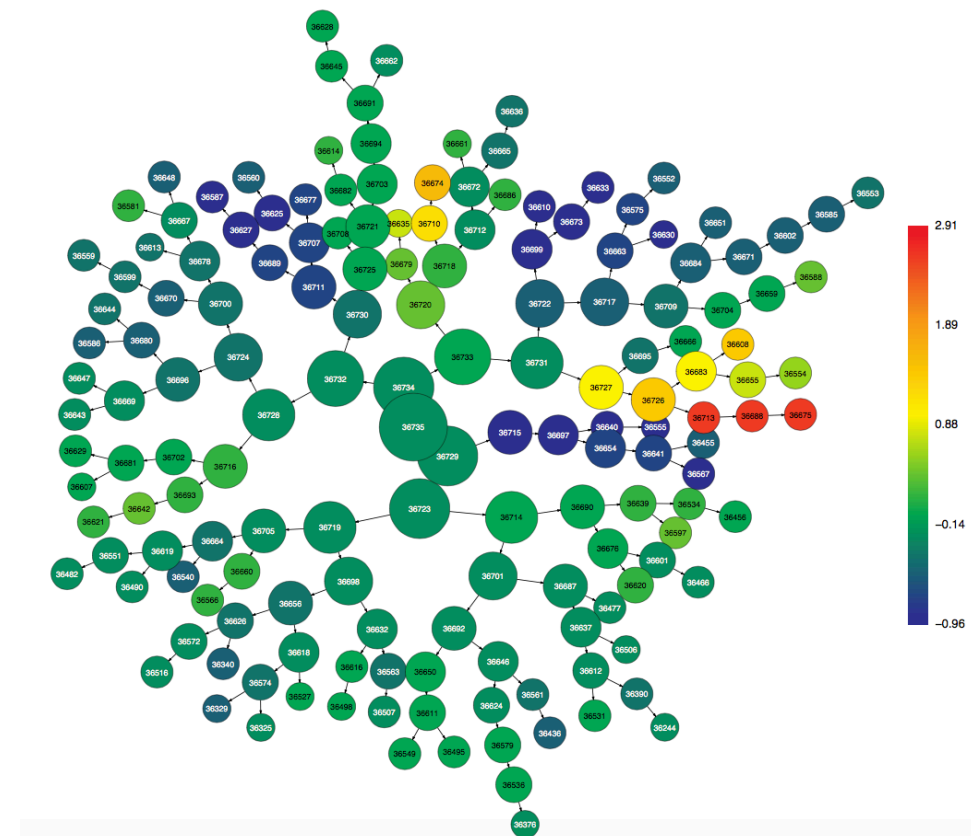
I:

PD-1



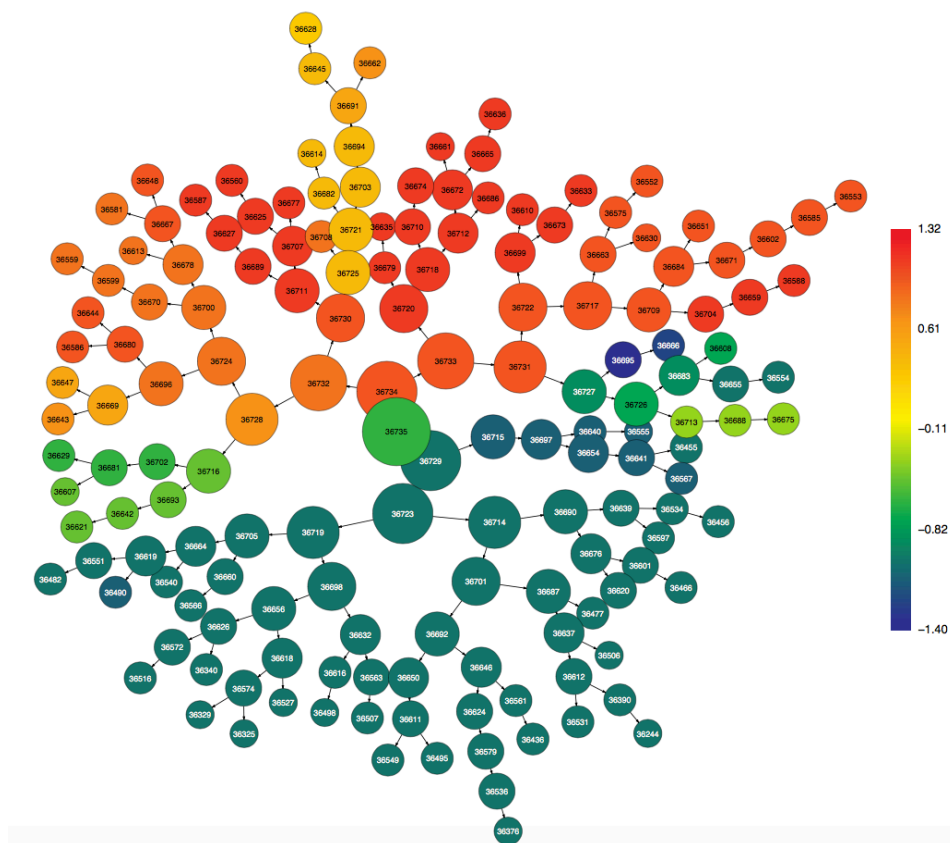
J:

Tim-3



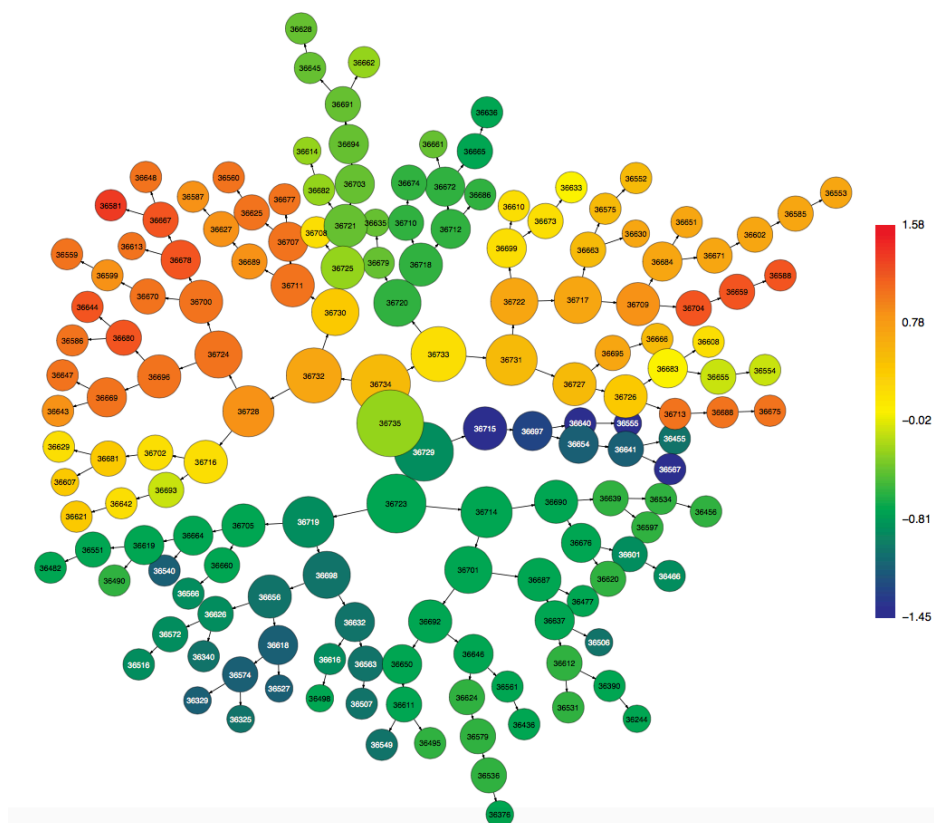
K:

CD45RA



L:

CCR7



M:

CD27

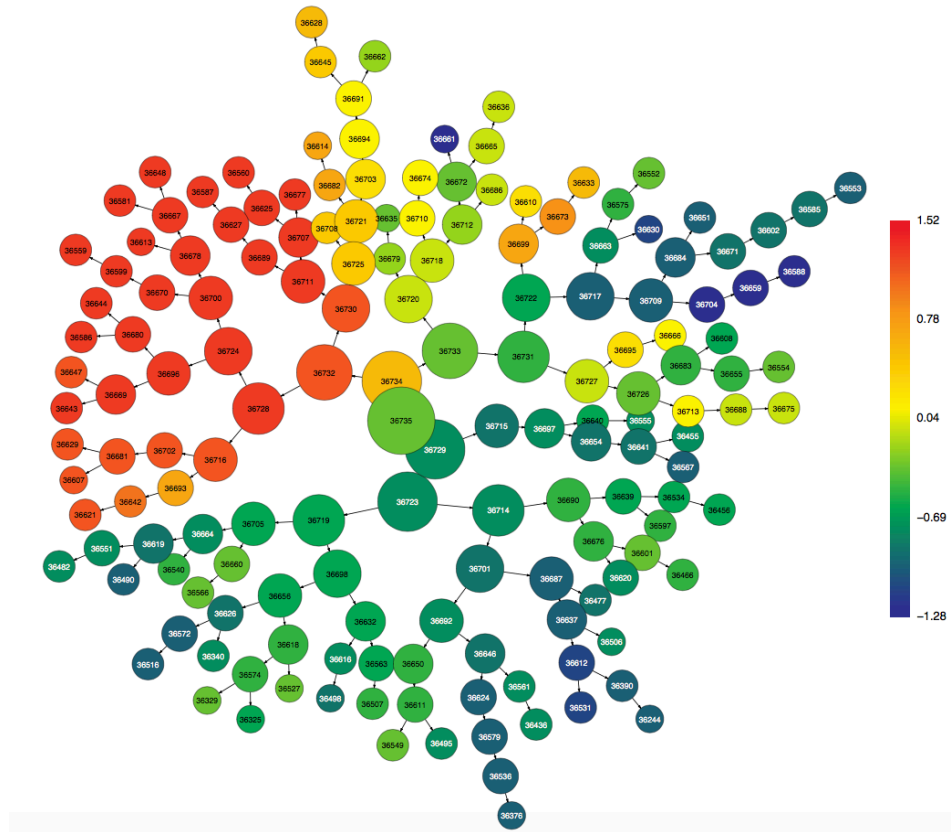


Figure 5-3: Hierarchical-clusters plots coloured by the median level of clustering markers expression in **A)** CD3, **B)** CD4, **C)** CD8, **D)** CD56, **E)** 2B4, **F)** CTLA-4, **G)** KLRG-1, **H)** TIGIT, **I)** PD-1, **J)** Tim-3, **K)** CD45RA, **L)** CCR7, and **M)** CD27. All the plots demonstrate the same clustering hierarchy. The cluster in the middle of the plot entitled “36735” is the “ancestor” node containing all the events in CITRUS analysis. Each node divided into 1 or 2 smaller nodes towards the periphery are the “descendant” nodes. Brighter colours correlate with higher levels of marker expression in each plot.

CITRUS consists of multiple algorithmic models for the analysis of different groups of samples. CITRUS first plots hierarchical clusters in order to clustering the cells into populations that help us to predict the significant differences of expression of markers on differential subsets among different groups. In our studies, we intend to characterize the potential differences of inhibitory receptors expression on peripheral T cells among 4 experimental groups.

In the CITRUS plot for our analysis, every node in the plot has a node number that is the tag to distinguish different clusters of population and a reference for further analysis. All the data of 66 individuals were gated on “live cells” (negative population of “live/dead staining”), which is primary “ancestor node”. In Figure 5-3, node-36735 in the middle of the plot corresponds to “live cells” equally obtained from all the data after mathematical computation.

The logistics of clustering hierarchy in a CITRUS plot is that every ancestor node usually divides into 2 descendant nodes and so on towards the periphery. For example, in Figure 5-3, the ancestor node-36735 split into node-36734 and node-36729. Further, node-36734, as the secondary ancestor node split into node-36732 and node-36733. Therefore, CITRUS plot redundantly represents events acquired from all research subjects in the hierarchical tree. Specifically this means all the events in node-36733 are contained in node-36734 and all the events in node-36734 are contained in node-36735.

In the analysis we have also set the minimum cluster size at 367 events of a node (1% of total plotting events). Hence, when the hierarchy of the tree is approaching to the threshold of 367 events, one ancestor node may split into only one descendant node. For example, in Figure 5-3, node-36645 split into node-36628 only. Since all the nodes were clustered on the basis of all the functional markers and subset markers we have chosen in 14-color panel, this hierarchical clustering logistics would enable us to identify the rare population that may contribute to the progression of chronic HBV associated diseases. Again, this concept was illustrated in Figure 5-3.

A CITRUS plot reveals not only the expression of inhibitory receptors on conventional lymphocytic subsets but also that on unknown or poorly defined lymphocytic subpopulations. From, Figure 5-3, A, to D, we are able to distinguish the conventional subsets of CD4+T cells, CD8+ T cells and NK cells in CITRUS plots based on the expression of CD3, CD4, CD8 and CD56. Next, we could also observe the expression of 6 inhibitory receptors (2B4, CTLA-4, KLRG-1, PD-1, Tim-3 and TIGIT) in Figure 5-3, E to J, and 3 T-cell differentiation markers (CD45RA, CCR7 and CD27) in Figure 5-3, K to M, on those conventional lymphocytic subsets. Specifically, node-36732 is the T cells cluster. Further divisions indicate that node-36730 is the CD8+ T cells cluster and node-36728 is the CD4+ T cells cluster. In non-CD3 subpopulations, node-36718 is the NK cells cluster. This is consistent to the discoveries by common methods that apply progressive gating for the analysis of the expression inhibitory receptors on T cells.

However, a CITRUS plot also enables us to simultaneously investigate the markers expression on unconventional or undefined lymphocytic subsets, which may elucidate crucial information in specific lymphocytic subsets in HBV associated disease progression. In Figure 5-3-D, node-36650 and node-36637 that are non-T cells, NK cells lineages exhibited similar 2B4 expression as observed in node-36696 that is the cluster of CD4+ T cells. In node-36731 that is a non-T cells, non-NK cells subset, a similar expression of CTLA-4 was detected when comparing CTLA_4 expression on T cells lineages. Interestingly, node-36713, the tertiary descendant of node-36731 demonstrated higher expression of CTLA-4 than that in its ancestor nodes and its expression is even much higher

than that in T cells. This indicates that the phenotype of the subset contained in node-36713 is masked in its ancestor clusters (node-36726, node-36727 and node-36731). In Figure 5-3, G-J, it is notable that node-36713 also co-expresses high expression of KLRG-1, PD-1, TIGIT and Tim-3. In addition, another discovery suggested that CD45RA, as one of the T-cell differentiation markers that enable us to distinguish naïve T cells from antigen experienced T cells, has a unique expression pattern in CD8+ T-cells lineages. In CD45RA positive CD8+ T cells, (node-36730), 2 lineages were subdivided by the differential intensity of CD45RA expression. Node-36725 demonstrated dim CD45RA positive expression, however, node-36711 exhibited bright CD45RA expression. These 2 lineages of CD8+ T cells may have distinctive roles in T-cells responses against HBV virus and cancer.

In conclusion, this set of data has several implications. Firstly, based on the rationale of CITRUS algorithms, it split all the events into hierarchical clusters by the expression of multiple markers. Such an approach enables us to identify the rare population that is poorly defined or to characterize the subset that was masked in the conventional analysis. Secondly, CITRUS also provides the platform to simultaneously compare all interested markers from all the PBMCs. This has a critical implication, especially in the application of blockade immunotherapy, as the inhibitors are capable of binding to other non-effector subsets that express inhibitory receptors. Blocking those non-effector subsets that have different immunological roles may induce potential adverse effects.

However, a CITRUS plot also enables us to simultaneously investigate the markers expression on unconventional or undefined lymphocytic subsets, which may elucidate crucial information in specific lymphocytic subsets in HBV associated disease progression. In Figure 5-5-D, node-36650 and node-36637 that are non-T cells, NK cells lineages exhibited similar 2B4 expression as observed in node-36696 that is the cluster of CD4+ T cells. In node-36731 that is a non-T cells, non-NK cells subset, a similar expression of CTLA-4 was detected when comparing CTLA_4 expression on T cells lineages. Interestingly, node-36713, the tertiary descendant of node-36731 demonstrated higher expression of CTLA-4 than that in its ancestor nodes and its expression is even much higher than that in T cells. This indicates that the phenotype of the subset contained in node-36713 is masked in its ancestor clusters (node-36726, node-36727 and node-36731). In Figure 5-5, G-J, it is notable that node-36713 also co-expresses high expression of KLRG-1, PD-1, TIGIT and Tim-3. In addition, another discovery suggested that CD45RA, as one of the T-cell differentiation markers that enable us to distinguish naïve T cells from antigen experienced T cells, has a unique expression pattern in CD8+ T-cells lineages. In CD45RA positive CD8+ T cells, (node-36730), 2 lineages were subdivided by the differential intensity of CD45RA expression. Node-36725 demonstrated dim CD45RA positive expression, however, node-36711 exhibited bright CD45RA expression. These 2 lineages of CD8+ T cells may have distinctive roles in T-cells responses against HBV virus and cancer.

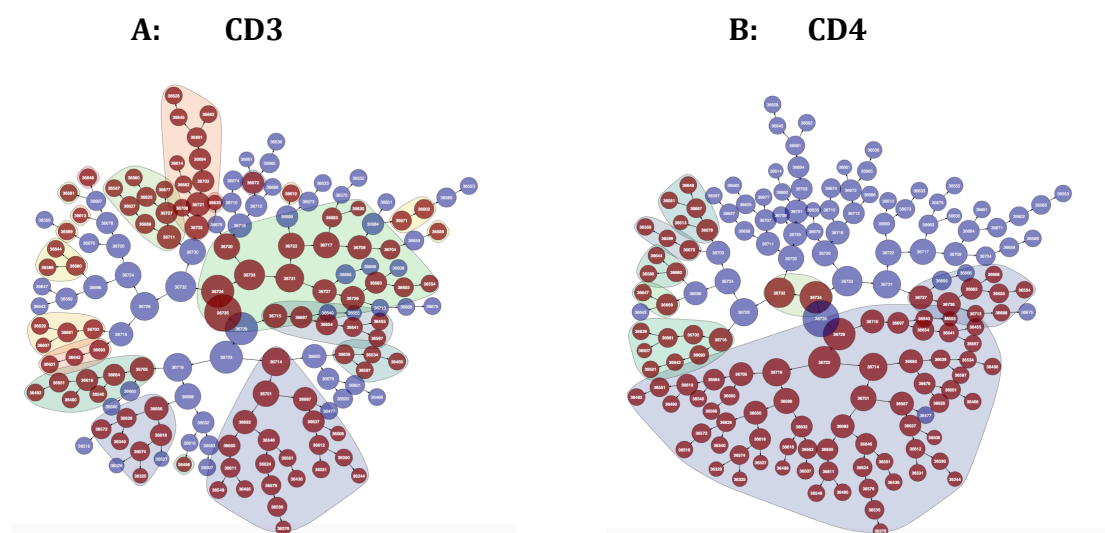
In conclusion, this set of data has several implications. Firstly, based on the rationale of CITRUS algorithms, it split all the events into hierarchical clusters by the expression of multiple markers. Such an approach enables us to identify the rare population that is poorly defined or to characterize the subset that was masked in the conventional analysis. Secondly, CITRUS also provides the platform to simultaneously compare all interested markers from all the PBMCs. This has a critical implication, especially in the application of blockade immunotherapy, as the inhibitors are capable of binding to other non-effector subsets that express inhibitory receptors. Blocking those non-effector subsets that have different immunological roles may induce potential adverse effects.

5.2.2.2 CITRUS analysis predicts subsets that exhibit significant phenotypes in different disease courses

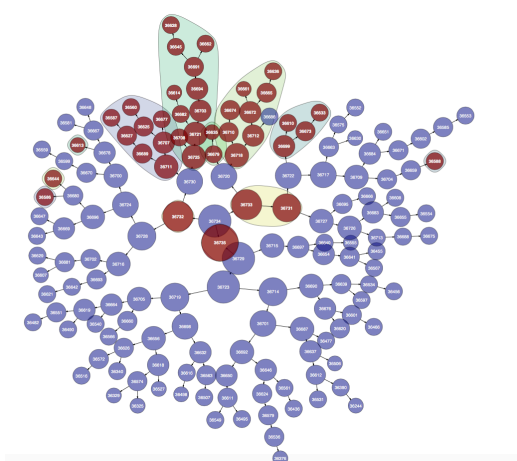
As the CITRUS plots have split events into hierarchical clusters on the basis of the expression of markers, the next question is, which subset would exhibit significantly differential expression among the 4 experimental groups. Therefore, the second part of the CITRUS process was programmed to predict subsets under each marker expression that may demonstrate distinctive expression in all the experimental groups. The CITRUS feature-plots were illustrated in Figure 5-6 and nodes that have statistical significance for the 13 markers including CD3, CD4, CD8, CD56, 2B4, CTLA-4, KLRG-1, TIGIT, PD-1, Tim-3, CD45RA, CCR7 and CD27, were highlighted in red. Generally, multiple nodes that may have significance among all the experimental groups under the predictive models were revealed in the expression level of each marker. Combining the results that we have interpreted from the CITRUS plot in Figure 5-3, it is straightforward to

determine which lymphocytic lineages those nodes belong to. As we focused on studies of CD8+ T cells, and then narrowed down our focus to select the interesting subsets in order to better answer our research question. Hence, nodes that share predictive significance in both CD3 and CD8 expression were selected. Overall, the co-significance nodes under CD3 and CD8 CITRUS-feature plots could be stratified into 2 phenotypic subpopulations; 1: CD3+CD8+CD45RA^{high} subset (node-36711 and its descendant nodes); 2: CD3+CD8+CD45RA^{low} subset population (node-36725 and its descendant nodes). Since CD8+ T cells is a highly heterogeneous population, the strategy to choose a predictive node for validation should start from the nodes in the periphery of the CITRUS plot as those are the clusters that may carry phenotypic significance. Therefore, node-36614 was chosen for further investigation.

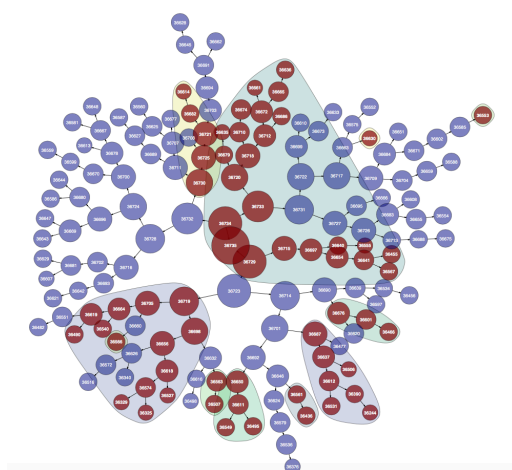
Figure 5-4: CITRUS feature-plots generating prediction of phenotypically distinctive subsets among different experimental groups



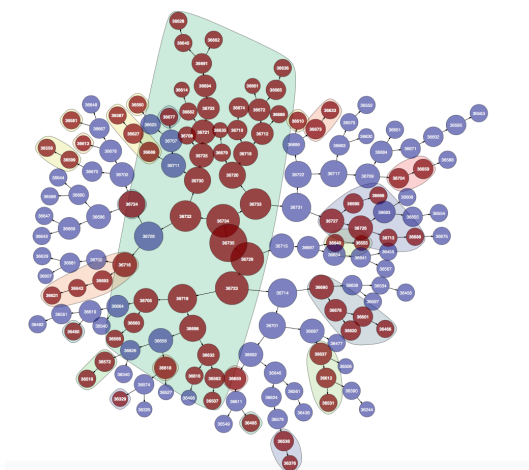
C: CD8



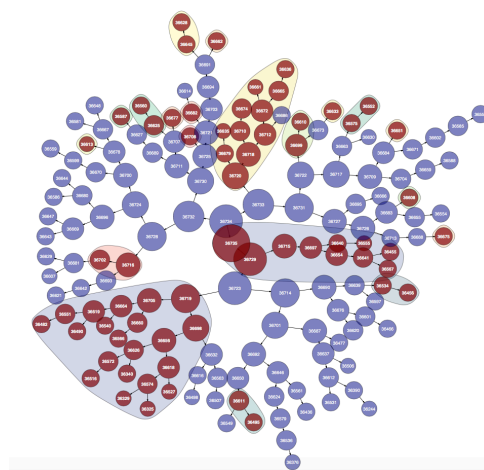
D: CD56



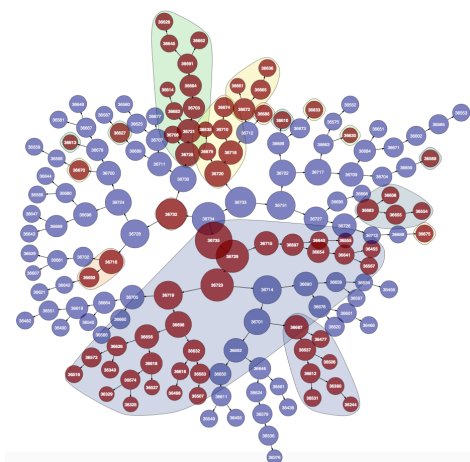
E: 2B4



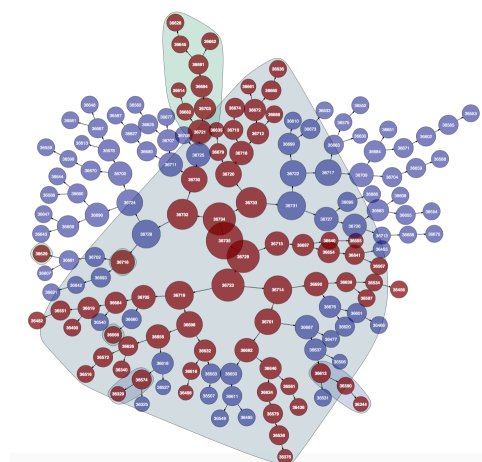
F: CTLA-4



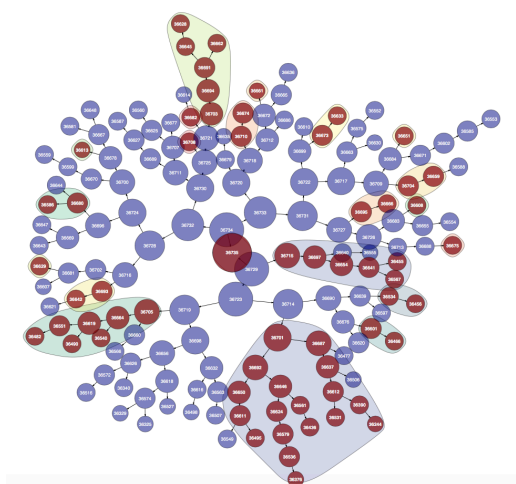
G: KLRG-1



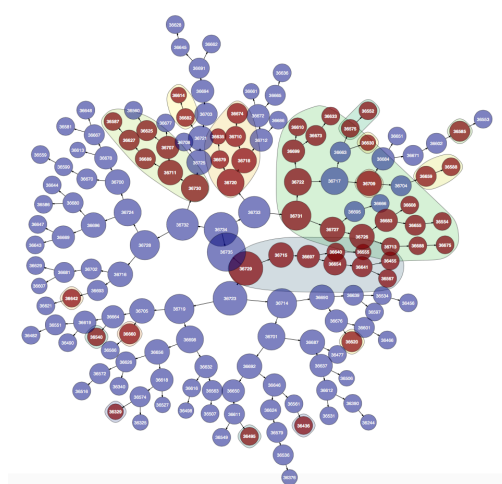
H: TIGIT



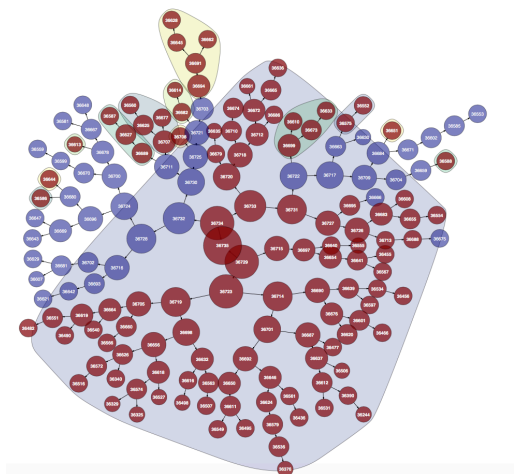
I: PD-1



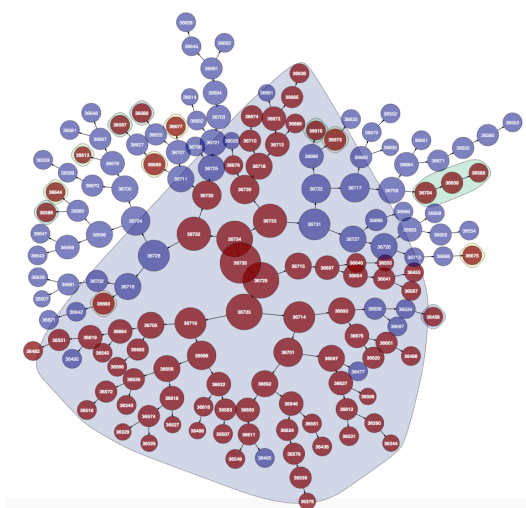
J: Tim-3



K: CD45RA



L: CCR7



M: CD27

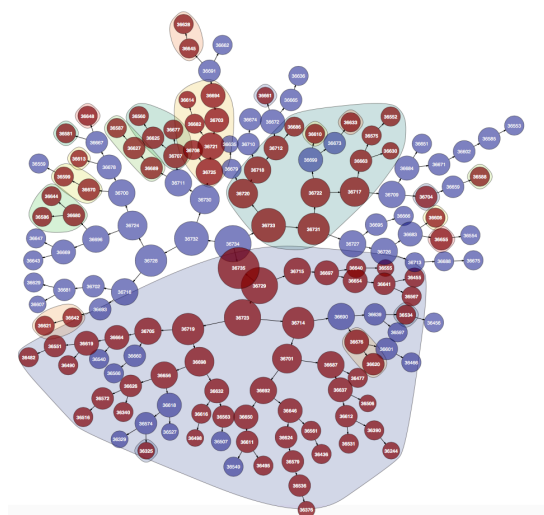
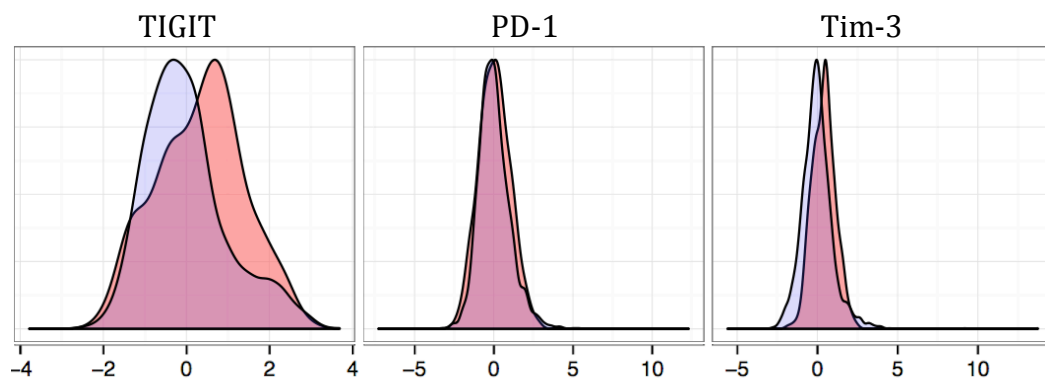


Figure 5-4: Hierarchical-clusters feature plots calculated by the CITRUS algorithm to predict differential expression subsets among healthy donors, CHB, HBV associated cirrhosis and HBV associated HCC in **A)** CD3, **B)** CD4, **C)** CD8, **D)** CD56, **E)** 2B4, **F)** CTLA-4, **G)** KLRG-1, **H)** TIGIT, **I)** PD-1, **J)** Tim-3, **K)** CD45RA, **L)** CCR7, and **M)** CD27. Statistical significance nodes among the 4 experimental groups were highlighted in red. Combining results from Figure 5-5, target subsets could be selected for further investigation based on the research questions and experimental hypothesis.

Moreover, to precisely predict the subsets that may display distinctive phenotypes among the 4 experimental groups, additional CITRUS algorithms were then applied to plot the histograms of clusters in order to characterize the phenotype of target subsets. All the histograms of nodes clustered in the CITRUS plot are attached in Appendix-5. Node-36614, one of the terminal clusters of CD3+CD8+ branch was analyzed. Apart from CD3 and CD8 expression, histograms of other markers were plotted to characterize the phenotype of this cluster (Figure 5-5).

Figure 5-5: Histograms of node-36614 in CITRUS plot identifies the phenotype of subset it represents



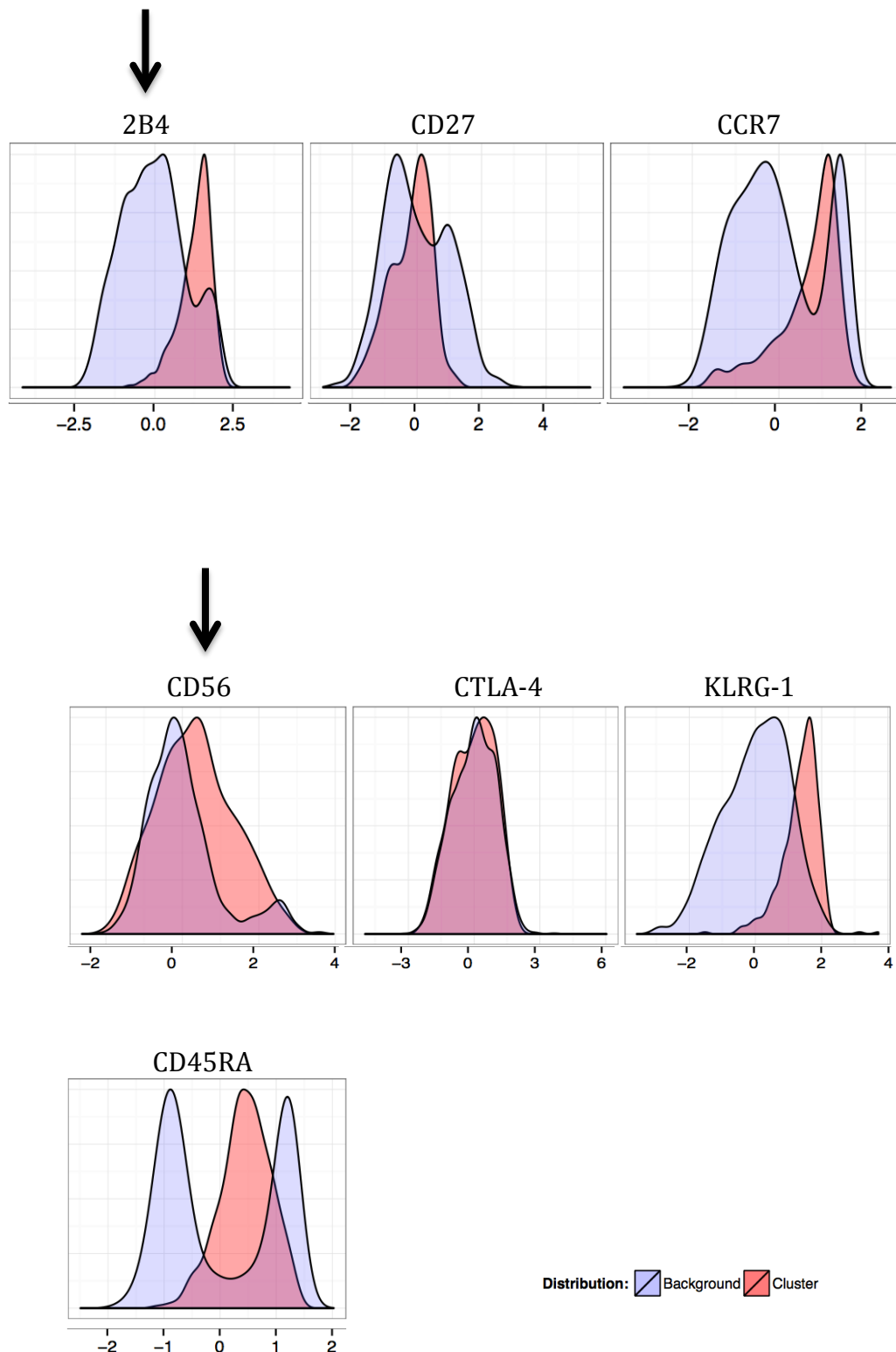
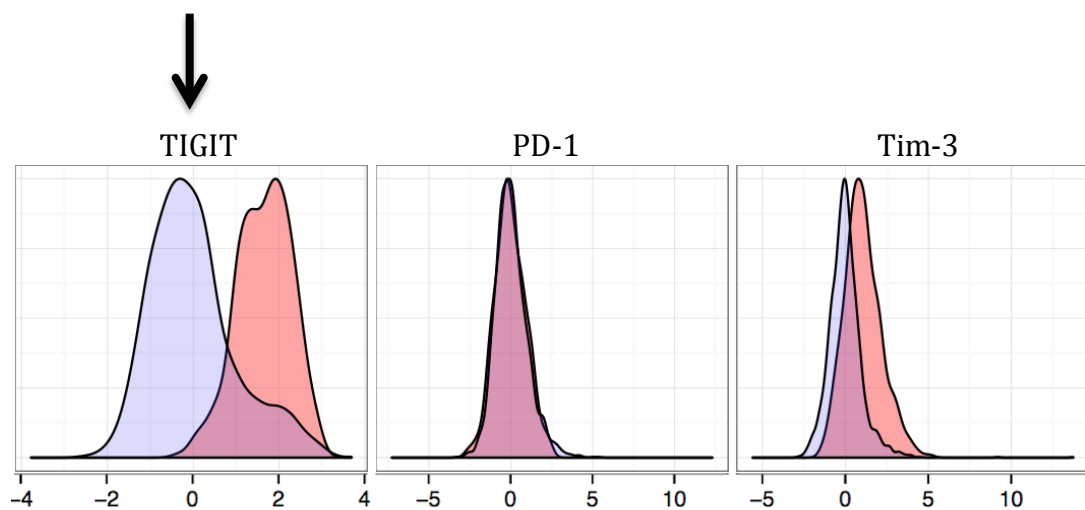


Figure 5-5: Histogram of 10 markers in node-36614 apart from CD3 and CD8 were plotted in CITRUS algorithms. It indicated that this cluster may phenotypically exhibit 2B4 and CD56 that subset may demonstrate differential frequency among healthy donors, CHB, HBV cirrhosis and HBV associated HCC. The blue histogram is the background and the red histogram is the marker expression of the cluster. Black arrows point out the positive expression of 2B4 and CD56 on node-36614.

In such an approach, the predictive phenotype of node-16614 is CD3+CD8+CD56+, which is possibly a subset belonging to NK T cells as it co-expresses marker of T cells (CD3) and marker of NK cells (CD56). It indicated that 2B4 may exhibit differential expression in CD3+CD8+CD56+ subset among all the experimental groups. In addition, another cluster (node-36635) in CITRUS stood out, as it is in the periphery of the CD3-CD56+ branch, but co-expresses CD8. Furthermore, in Figure 5-4, 5 out of 6 inhibitory receptors in the CITRUS feature-plots indicated node-36635 may exhibit differential expression among the 4 experimental groups. Hence the same histograms as in were plotted by CITRUS to characterize the phenotype of this subset in Figure 5-6.

Figure 5-6: Histograms of node-36635 in CITRUS plot identifies the phenotype of subset it represents



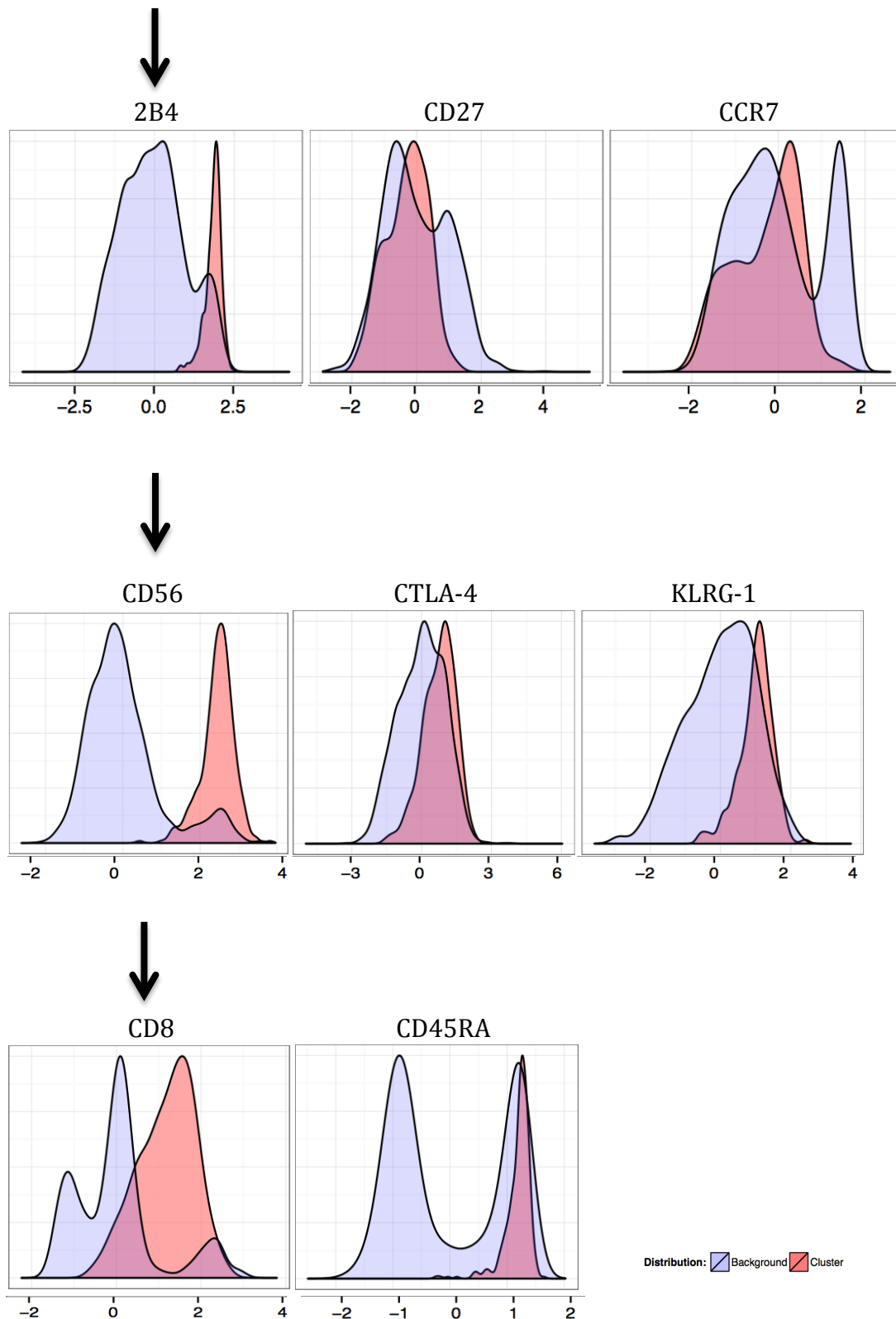


Figure 5-6: Histogram of 11 markers in node-36635 were plotted in CITRUS algorithms. It indicated that this cluster may phenotypically exhibit 2B4, TIGIT, CD8 and CD56 that the subset may demonstrate differential frequency between 4 experimental groups. Blue histogram the background and red histogram is the marker expression of the cluster. Black arrows point out the positive expression of TIGIT, 2B4, CD56 and CD8 on node-36635.

From Figure 5-5, the predictive phenotype of node-36635 is CD3-CD8+CD56+. This subset is a terminal branch of the CD3-CD56+ cluster, which is an NK cell phenotype, but it co-expresses 2B4 and TIGIT. Since 2B4 and TIGIT are inhibitory receptors we investigated, these results suggested that CD3-CD56+CD8+ subset may demonstrate distinctive co-expression of 2B4 and TIGIT within all experimental groups. Hence by using the CITRUS algorithms, we narrowed down our investigation to 2 subsets that may demonstrate differential sole expression or co-expression of inhibitory receptors originated from 2 broad branches of NK T cell and NK cell population within all experimental groups. Next critical question is to determine and validate 2 subsets that may exhibit distinctive phenotypes within the 4 experimental groups. This would be validated by an independent analysis that was not conducted in CITRUS to avoid the false positive discovery.

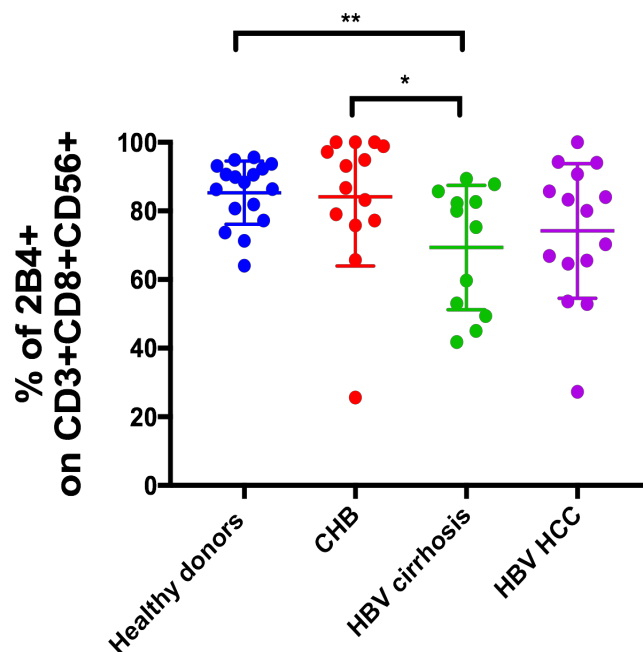
In conclusion, applying CITRUS analysis in the prediction of subsets may facilitate the identification of subsets that exhibit distinctive expression of inhibitory receptors within the 4 experimental groups. This enables us to focus on subsets that may have the potential to be the biomarkers or may act prominent roles in disease progression. In addition, that CITRUS systemically characterizes subsets in hierarchical clustering manner greatly assists analysis of high-dimensional flow cytometric data while providing additional information.

5.2.2.3 Validation of subsets predicted by CITRUS that may demonstrate differential expression among different experimental groups

66 individuals ranging from healthy donors (16/66), CHB (14/66), HBV associated cirrhosis (11/66) and HBV associated HCC (15/66), were analyzed in an independent file by using the conventional analysis method in Flowjo. Subsets were gated according to the predictive phenotype of node-36614 and node-36635. Node-36614 was gated on CD3+CD8+CD56+2B4+ population and node-36635 was gated on CD3-CD56+CD8+2B4+TIGIT+ population using the progressive gating strategy. Dot plots are depicted in Figure 5-6 and Figure 5-7 to compare the frequency of 2 subsets within the 4 different experimental groups respectively.

Figure 5-6: Differential expression of 2B4 in CD3+CD8+CD56+ subset among 4 experimental groups

A:



B:

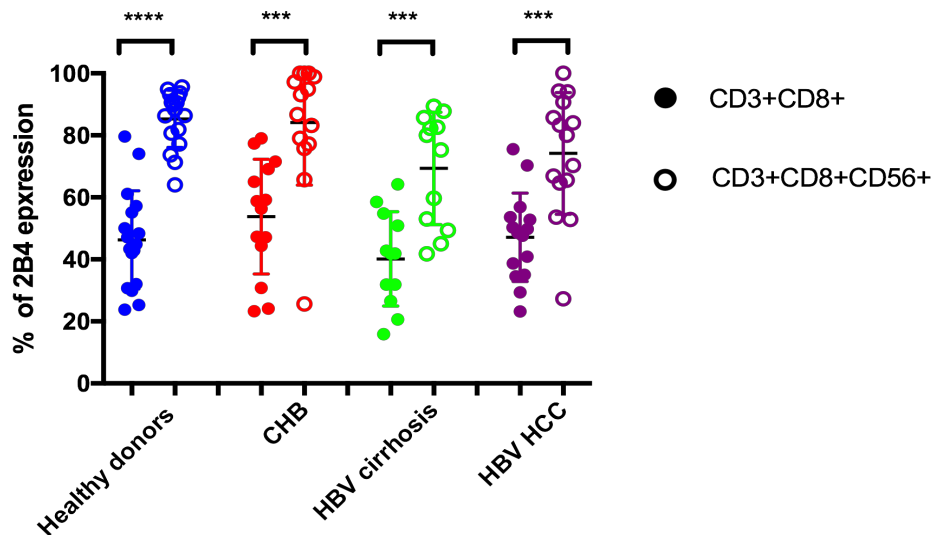


Figure 5-6: A) Expression analysis of 2B4 (in %) in CD3+CD8+CD56+ subset among healthy donors, CHB individuals, HBV associated cirrhosis individuals and HBV associated HCC individuals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney t-test). **B)** Comparative analysis of 2B4 (in %) expression between CD8+ T cells and CD3+CD8+CD56+ subset among healthy donors, CHB individuals, HBV associated cirrhosis individuals and HBV associated HCC individuals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, paired t-test).

Results from Figure 5-6-A demonstrated that 2B4 has a lower expression in CD3+CD8+CD56+ subset in HBV associated cirrhosis individuals when compared to that of CD3+CD8+CD56+ subset in CHB individuals and healthy donors. Meanwhile, results from Figure 5-6-B revealed that 2B4 has a significantly higher expression in the CD3+CD8+CD56+ subset when compared to that of CD8 T within the 4 experimental groups.

This observation indicates that CD3+CD8+CD56+ as a subset belongs to NK T cells, may downregulate 2B4 expression in the progression from CHB to HBV associated cirrhosis. Human livers have relatively lower frequency of NK T cells than mouse livers. It is rather difficult to characterize the complex role of NK T cells in virus controls and fibrogenesis. Several studies indicate that activated

NK T cells may promote liver fibrosis by releasing cytokines of IL-4 and IL-13, hedgehog ligand and osteopontin[259]. However, other studies also suggested NK T cells could also attenuate liver fibrosis by producing INF-gamma, perforin and FASL by which it induces apoptosis of hepatic stellate cells (HSC) that are a key subset in the liver facilitating the development of liver fibrosis[260, 261].

Consequently, despite no intracellular staining being performed to characterize the functional signatures of this subset, it is rational to hypothesize that the downregulation of 2B4 on CD3+CD8+CD56+ subset may represent an activated state of NK T cells subset with enhanced fibrogenic cytokines production as it is only observed within the HBV associated cirrhosis group but not in the CHB group. Therefore, lower expression of one of the inhibitory receptors, 2B4 in NK T cells subset, may have a critical role in disease progression of chronic HBV infection and exert an important role in liver fibrosis and the establishment of cirrhosis. This would be a valuable subset for further investigation. In addition, results from Figure 5-6-B indicate that 2B4 expression on the CD3+CD8+CD56+ subset was generally masked in CD8 T cells in all 4 experimental groups.

Therefore, CITRUS analysis facilitates the identification of characterization of this subset from bulk CD8 T cells. It also implies that 2B4 expression on NK T cells subset may serve as a self-tolerance mechanism as it has a higher expression in CD3+CD8+CD56+ subset when compared with bulk CD8 T cells from healthy individuals. Hereafter, characterizing the expression of inhibitory receptors on other effector subsets, such as NK T cells or NK cells, may have

predictive values in the evaluation of adverse effects of blockade immunotherapies.

Next the same validation was applied on another cluster node-36635 that was predicted by CITRUS analysis. This cluster is a terminal branch from CD3-CD56+ cells but co-expresses low CD8 that belongs to the NK cells subset. Based on the prediction, we then analysed the co-expression of 2B4 and TIGIT on CD3-CD56+CD8+ subset in Figure 5-7.

Figure 5-7: Differential co-expression of 2B4 and TIGIT in CD3-CD8+CD56+ subset within the 4 experimental groups

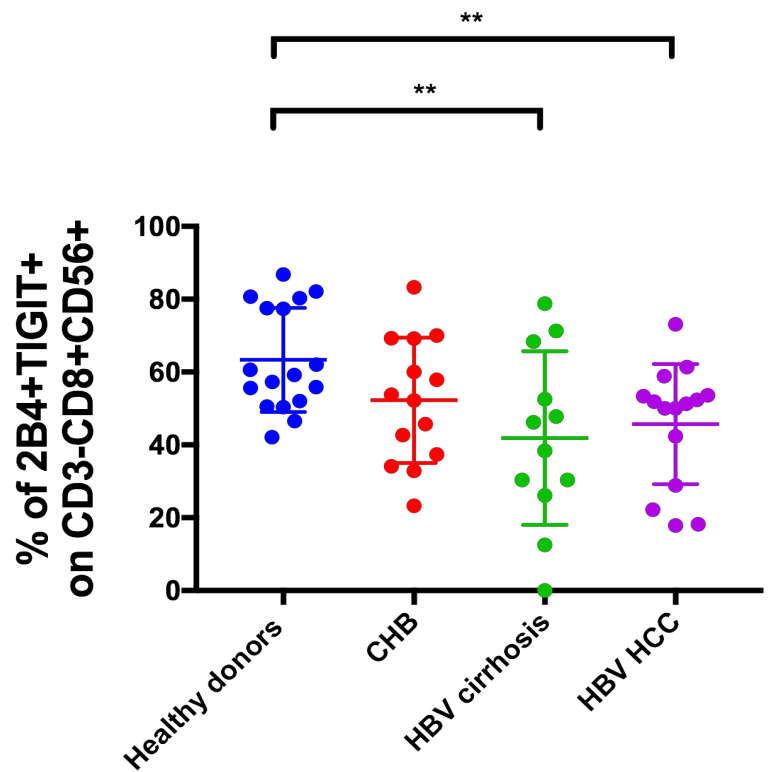


Figure 5-7: co-expression analysis of 2B4 and TIGIT (in %) in CD3-CD8+CD56+ subset among healthy donors, CHB individuals, HBV associated cirrhosis individuals and HBV associated HCC individuals (*p<0.05, **p<0.01, ***p<0.001, Mann-Whitney t-test).

In Figure 5-7, co-expression of 2B4 and TIGIT on CD3-CD56+CD8+ subset significantly decreased in HBV associated cirrhosis and HBV associated HCC individuals when compared with that on the CD3-CD56+CD8+ subset from healthy donors. Based on its phenotype, this subset belongs to NK cells. Generally, in chronic HBV infection, NK cells have been suggested to be a double-edge sword. On the one hand, it is capable of limiting liver fibrosis by killing HSCs in TRAIL or NKG2D manner[262]. And hepatic NK cells may also exert protective role of hepatocytes through the production of IL-22, which could limit tissues damage[263]. On the other hand, NK cells may contribute to the depletion of HBV specific CD8+ T cells in chronic HBV infection through the interaction between TRAIL on NK cells and TRAIL-R2 death inducing receptor on HBV specific CD8+ T cells[264].

From our results in Figure 5-7, we have observed that the co-expression of 2 inhibitory receptors, 2B4 and TIGIT on CD3-CD56+CD8+ NK cells subset has been significantly decreased in HBV associated cirrhosis and HBV associated HCC (advanced stages of HBV chronic infection), when compared with that expression of CD3-CD56+CD8+ NK cells subset in healthy donors. Since inhibitory receptors also modulate NK mediated innate immunity, therefore, downregulation of 2B4 and TIGIT in CD3-CD56+CD8+ NK subset in HBV associated cirrhosis and HBV associated HCC may be a reflection of HBV specific CD8 T cells depletion through activated NK cells that phenotypically demonstrates lower co-expression of 2B4 and TIGIT, although no functional experiments on this subset were conducted. This set of data, it indicates that when applying inhibitory pathway blockade immunotherapy to treat chronic

HBV infection or HBV associated HCC, we should pay attention to the activation of NK cells as a “side effect” as it may augment the depletion of antigen specific CD8+ T cells. In addition, this result also independently validates the identification of novel subsets that may demonstrate expression of differential markers within the 4 experimental groups by using CITRUS analysis.

In conclusion, CITRUS analysis enables us to systemically analyse high dimensional flow cytometric data using hierarchical clusters. This study allows us to identify the differential expression of 2B4 in the CD3+CD8+CD56 subset within 4 experimental groups. Furthermore, it also identifies the differential co-expression of 2B4 and TIGIT in the CD3-CD8+CD56+ subset within 4 experimental groups. Those discoveries may help us to elucidate the regulation of inhibitory receptors on different subsets in a chronic virus infection or cancer. CITRUS as a newly developed programming language was tailored to investigate mass cytometry data and was firstly published in 2014[265]. But in our study, we have successfully applied it to fluorescent cytometric data in order to answer our research questions. This automated identification and characterization algorithm is a powerful tool to reveal the heterogeneity of a population from multiple experimental groups. In the second part of our experiments, further validation using independent conventional analysis authenticates CITRUS findings. In the near future, we intend to apply CITRUS to the heterogeneous expression of multiple inhibitory receptors on tumor-infiltrated lymphocytes from cancer individuals.

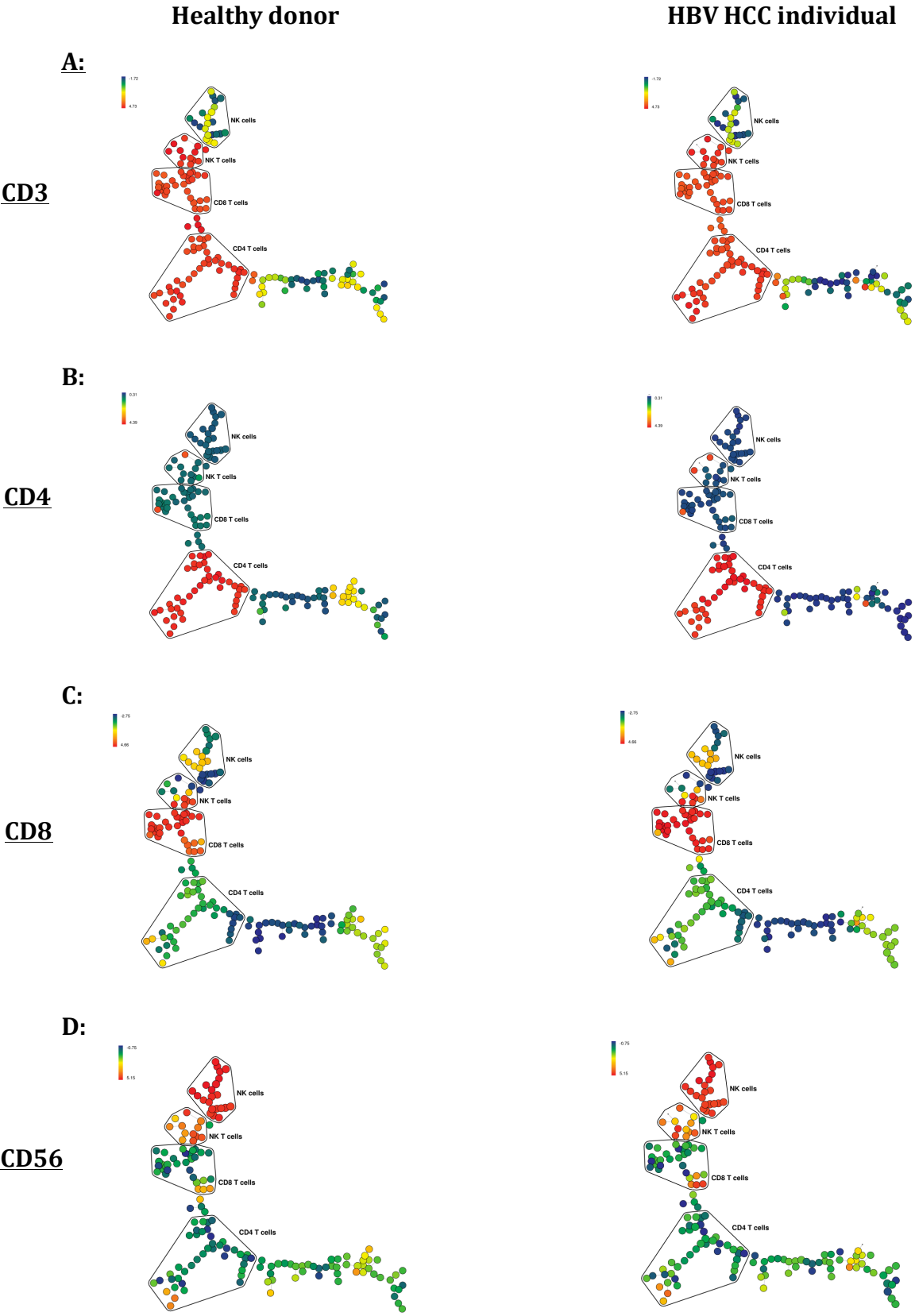
5.2.3 Applying SPADE algorithms for the expression analysis at individual level

SPADE (Spanning-tree Progression Analysis of Density-normalized Events) is a next generation computational analysis method that automatically identifies populations of interest from high dimensional flow cytometric data[266]. Similar to CITRUS, it was initially designed for analysis of multiplex data from mass cytometry. Compared to CITRUS algorithms, which identify differential expression signatures among different experimental groups, SPADE algorithms concentrate on the phenotypic characterization of individuals that may carry crucial information to visually observe rare populations at single-cell level.

SPADE is a useful complementary analysis tool when used in conjunction with CITRUS and conventional supervised analysis[267]. SPADE algorithms could be subdivided into 4 modules, 1: SPADE equalizes each marker expression in both rare and abundant density in different clusters. 2: SPADE algorithms cluster phenotypically similar cells into clouds on the basis of markers expression. 3: SPADE algorithms calculate the proportion of rare populations and abundant populations enabling both types of population to be represented in the SPADE trees. 4: SPADE plotting algorithms demonstrate the results in SPADE trees in an unsupervised manner[268]. SPADE has been applied to the analysis of in vitro studies at multiple time points for monitoring dynamic change of cytokines production[267]. It also enables automatic identification and visualization of different subsets in 2 dimensional SPADE tree maps[269].

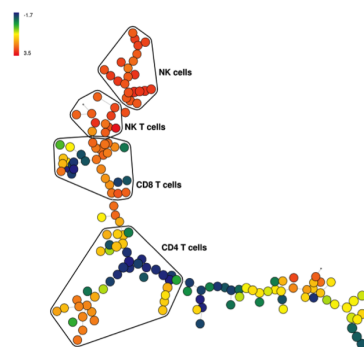
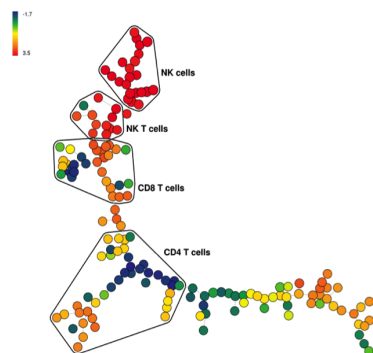
Hence, SPADE would be a potentially powerful tool to analyze expression patterns on whole lymphocytes from an individual research subject. SPADE analysis was used to compare PBMCs of 2 individuals from the abovementioned cohorts in which expression of 6 inhibitory receptors on T cells were analyzed. We aimed to simultaneously visualize the expression patterns of each inhibitory receptor in different subsets to compare data between individuals. In this comparison, one individual is a healthy donors, while the other is an HBV associated HCC patient. The SPADE trees are illustrated in Figure 5-8. Note: this Spade analysis uses the same raw data as was used in the CITRUS analysis.

Figure 5-8: SPADE trees reveal distinctive expression pattern of inhibitory receptors on different subsets in one healthy donor and one HBV HCC individual



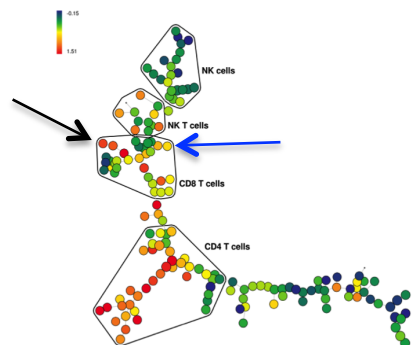
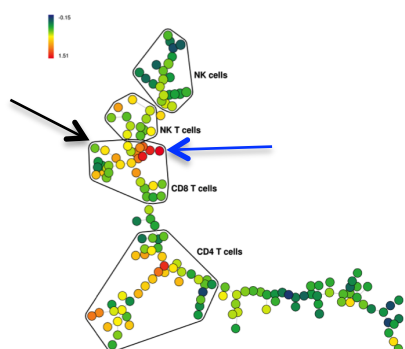
E:

2B4



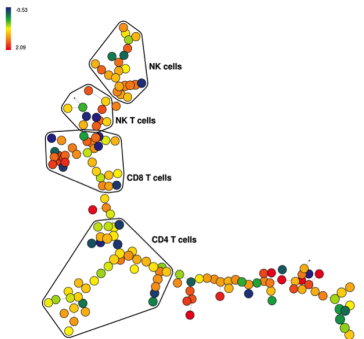
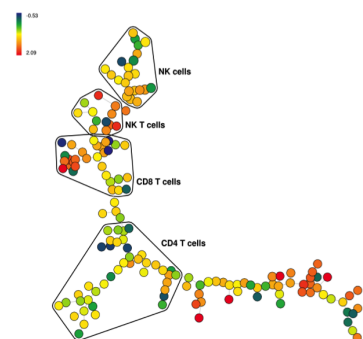
F:

PD-1



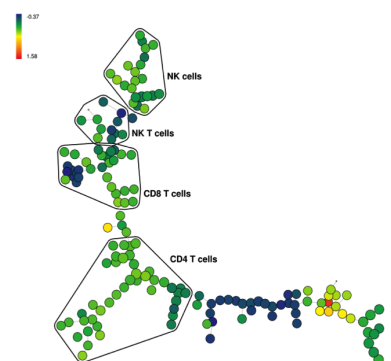
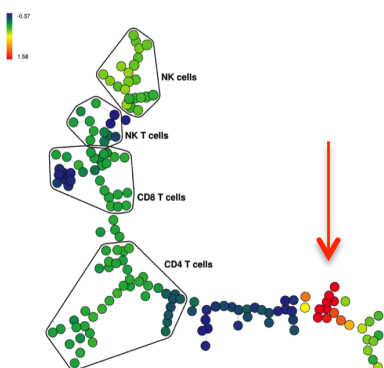
G:

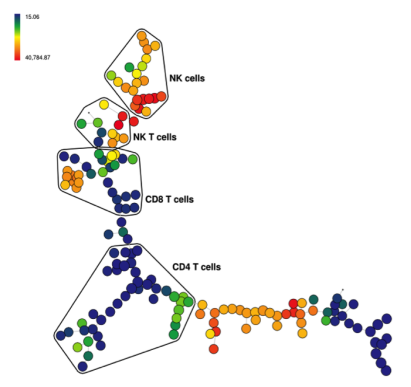
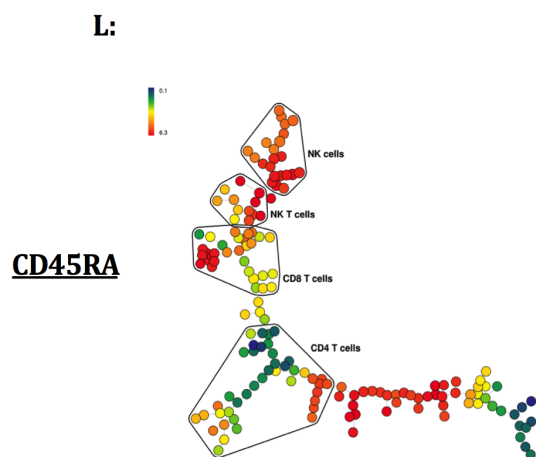
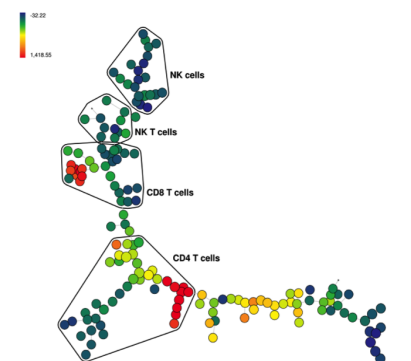
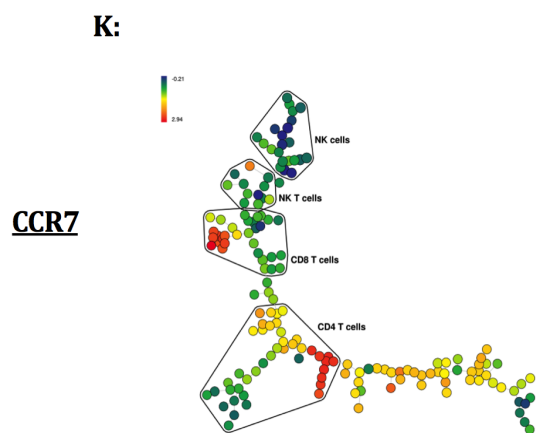
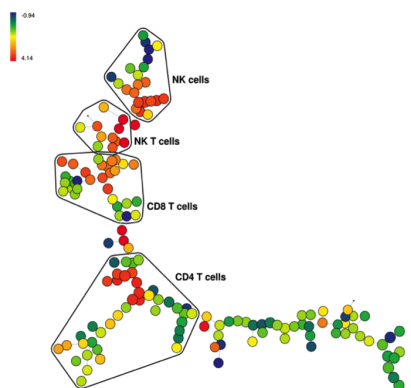
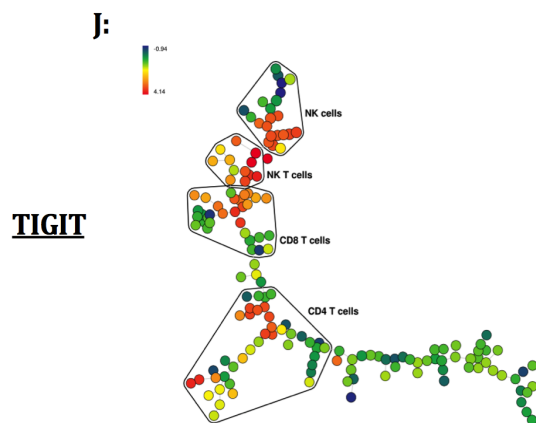
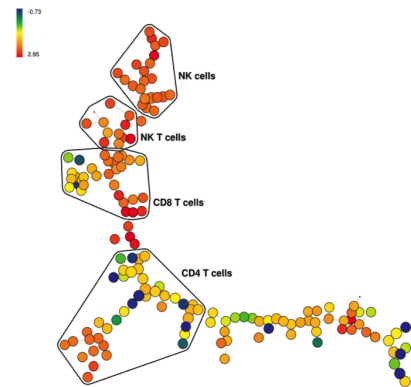
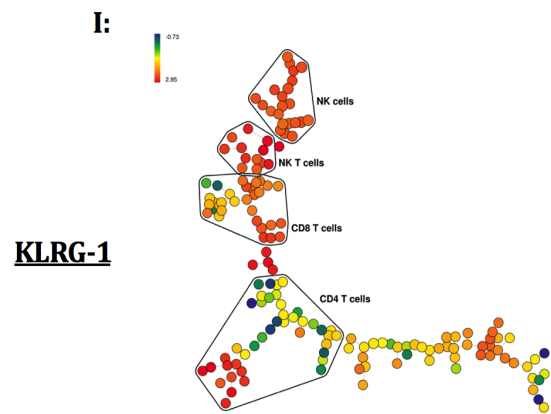
CTLA-4



H:

Tim-3





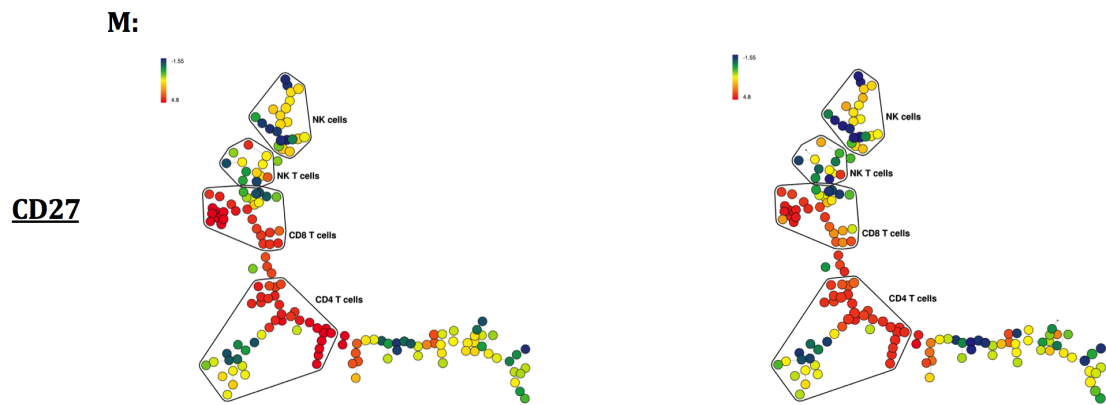


Figure 5-8: SPADE trees plotted to compare differential expression pattern of multiple markers between one healthy donor (left plot) and one HBV associated HCC individual (right plot). Equal number of cells was obtained from the flow data of each individual. Maximum cluster of each node in SPADE tree is 2672 cells. 200 nodes were plotted in each SPADE tree. SPADE trees were gated on “live cells”. Median fluorescent intensity of **A)** CD3, **B)** CD4, **C)** CD8, **D)** CD56, **E)** 2B4, **F)** PD-1, **G)** CTLA-4, **H)** Tim-3, **I)** KLRG-1, **J)** TIGIT, **K)** CCR7, **L)** CD45RA and **M)** CD27 was represented using color gradient.

In Figure 5-8 A-D, based on visualizing the expression of CD3, CD4 CD8 and CD56 on SPADE trees, CD4 T cells, CD8 T cells, NK T cells and NK cells were tagged by enclosing with black lines. No significantly differential expression of these 4 markers was observed in the comparison of the data from the PBMCs of the healthy donor and that of the HBV associated HCC individual. The undefined branch of clusters in the right corner of SPADE tree may represent other subsets including monocytes DCs and B cells. To characterize those subsets, additional markers are required. Interestingly, Tim-3 has a higher expression in the undefined subsets observed in healthy donor but not in HBV HCC individual (red arrow).

Next, in Figure 5-8 E-J, SPADE trees enable us to visualize the expression of 6 inhibitory receptors on different subsets, simultaneously, in 2 individuals. In the

comparison of 2B4 expression between 2 individuals (Figure 5-8 E), SPADE trees demonstrated that 2B4 expression was predominately on CD8 T cells, NK cells and NK T cells but not CD4 T cells in both individuals. But it has lower expression on NK cells and NK T cells in the HBV HCC individual when compared with the expression of those 2 subsets in the healthy donor.

In Figure 5-8 F, it is shown that PD-1 was expressed mainly on CD8 T cells and a small fraction of NK T cells while maintaining low expression in CD4 T cells in the healthy donor. However, in the comparison of high PD-1 expression subsets in the CD8 T cells branch, between healthy donor and HBV HCC individual, high PD-1 expression subsets in the CD8 T cells switched from 3 clusters (blue arrow) to another several clusters (black arrow) in the HBV associated HCC individual. Considering the results depicted in Figure 5-1 in this chapter that no differential expression of PD-1 on CD8 T cells was observed between healthy donors, CHB, HBV associated cirrhosis and HBV associated HCC. Although it is an individual based comparison, this result from SPADE trees may suggest that it has differential phenotypes of PD-1+ CD8 T cells between the healthy donor and the HBV associated HCC individual. In Figure 5-9, we further investigated the specific phenotypes of 2 clusters of PD-1+ CD T cells nodes. It was found that the major phenotypic differences between 7 PD-1+CD8 T cells nodes in the healthy donor and 3 PD-1+CD8 T cells nodes in the HBV associated HCC individual is the expression level of CD27 and CD45RA. Generally, in the healthy individual, the PD-1+ CD8 T cells mainly express CD45RA but not CD27. However, PD-1 +CD8 T cells in the HBV associated HCC individual mainly express CD27 but not CD45RA.

The expression of CD27 and CD45RA on T cells is linked with different stages of T-cells differentiation. CD27 is predominately expressed naïve T cells and lost its

Figure 5-9: Differential phenotypes of PD-1+CD8 cells between healthy donor and HBV associate HCC individual

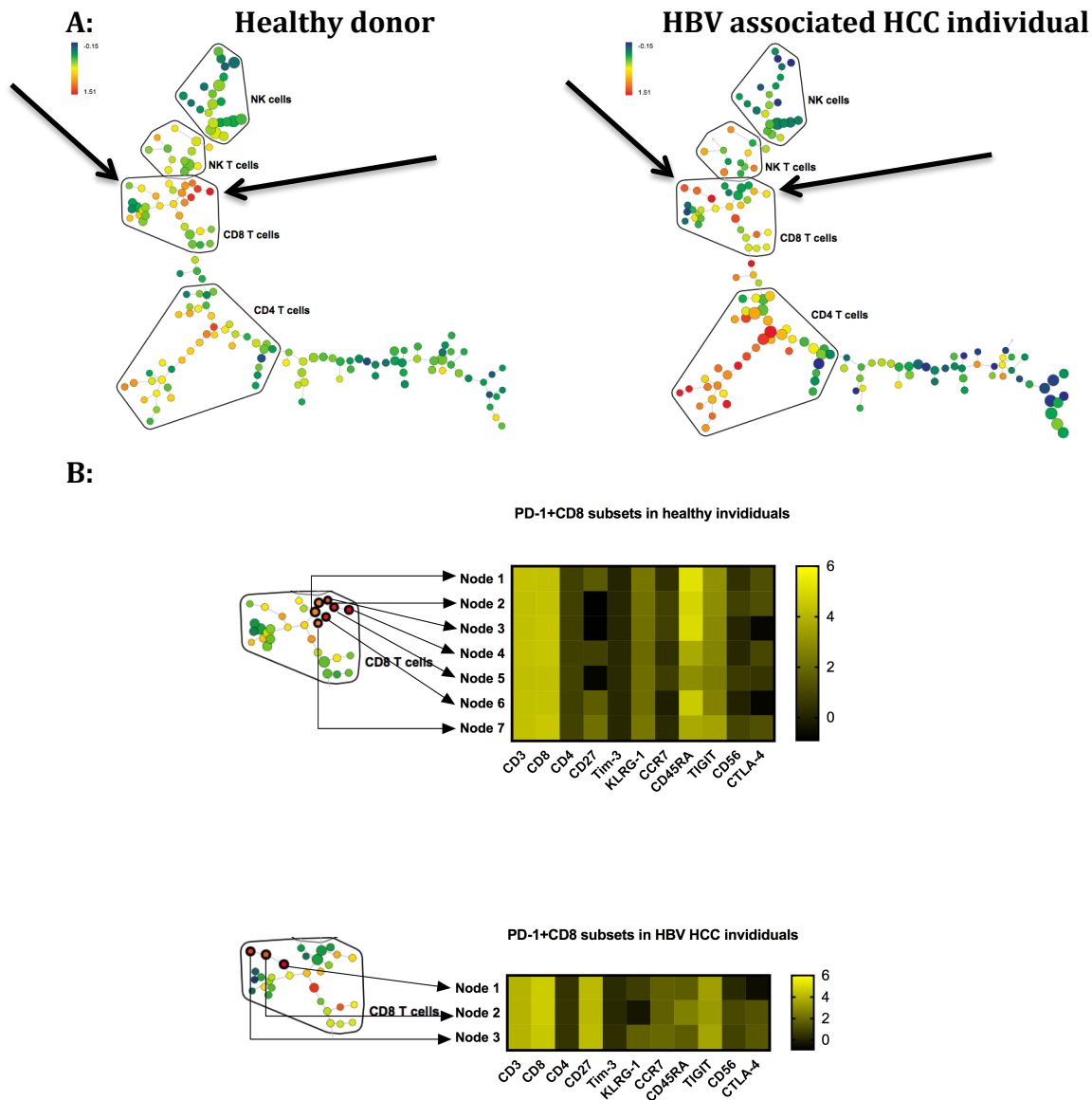


Figure 5-9: A: SPADE trees indicated differential phenotypes of PD-1+ CD8 T cells in peripheral blood between one healthy individuals and one HBV associated HCC individual. B: Heat maps drawn by the median fluorescent intensity of CD3, CD4 CD27, Tim-3, KLRG-1, CCR7,CD45RA, TIGIT, CD56 and CTLA-4 of 7 PD-1+CD8 T cells nodes in the healthy individual and 3 PD-1+CD8 T cells nodes in HBV HCC individual respectively.

expression through intermediate stage towards late stage of T cells differentiation[228, 229]. In the meanwhile, CD45RA is expressed on naïve T cells while losing its expression in intermediate and advanced T cells differentiation stages. However, it is re-expressed on late T cells differentiation stage[231].

In addition, from the results in Figure 5-9-B, it was shown that different PD-1+CD8 T cells nodes in the SPADE trees exhibited differential expression pattern of inhibitory receptors. In the healthy individual the differential expression pattern of inhibitory receptors among different nodes (node 1 to node 7) was mainly on the expression of TIGIT and CTLA-4. But in the HBV associated individual, the differential expression pattern of inhibitory receptors among different nodes (node 1 to node 3) was mainly on the expression of KLRG-1 and CTLA-4. This suggests the PD-1+CD8 T cells are a heterogeneous population that could be further divided into different phenotypes with distinctive functions.

The high PD-1 expression subset in the healthy donor may act as a normal immune-tolerance function, while the “switched” high PD-1 expression CD8+ subsets in HBV HCC individual may associate with T-cell exhaustion resulting from chronic HBV infection and the emergence of HCC. However, no differential expression of the PD-1 on entire CD8 T cells between the 2 individuals indicated that 2 PD-1+ CD8+ subsets were masked in the conventional analysis method but identified in SPADE analysis. Further characterization of 2 CD8 T cells subsets, mentioned above, is essential to elucidate its functions. In addition, the

HBV associated HCC individual has a higher PD-1 expression on CD4 T cells when comparing with that expression of CD4 T cells on the healthy donor, which may result from T-cells exhaustion.

In Figure 5-8 G, H, J, no significantly differential expression of CTLA-4, Tim-3 and TIGIT on CD4 T cells, CD8 T cell, NK cells and NK T cells was observed. But CTLA-4 has a wide expression on nearly all lymphocytic subsets, which might act in the role of normal immuno-tolerance function and may indirectly explain why the CTLA-4 inhibitor has severe adverse effects as the blockade affects a wide range of lymphocytes. In Figure 5-8 I, it is found that KLRG-1 was expressed mainly on CD8 T cells, NK T cells, NK cell and a fraction of CD4 T cells on both individuals. However, KLRG-1 has a comparatively lower expression on NK T cells and CD4 T cells in the HBV HCC individuals when compared with the healthy donor.

In Figure 5-8 K, M, no significantly differential expression of CCR7 and CD27 on both CD4 and CD8 T cells was detected between the 2 individuals. However in Figure 5-8 L, CD45RA has a lower expression on both CD4 and CD8 T cells in the HBV HCC individual when compared with the expression of both subsets in the healthy donor. CD45RA, as one of the markers that exhibit differential expression through T cells differentiation, has higher expression on naïve T cells while losing its expression upon differentiation but re-expresses on terminally differentiated T cells. Hence, the lower expression of CD45RA of T cells in the HBV HCC individual may result from persistent HBV virus infection and the establishment of cancer.

In conclusion, SPADE as a tool designed for mass cytometry, is capable of answering research questions using fluorescent flow cytometric data. Moreover, SPADE analysis enables us to investigate the expression of markers from high dimensional data into 2 dimensional SPADE trees at the single-cell level in an unsupervised manner. It allows us to compare and visualize the expression of markers in multiple subsets, simultaneously. In the above analysis, it provides additional information about the expression pattern of inhibitory receptors on different subsets, especially in the “switched” PD-1 high expression CD8 subsets. Through 2 individuals comparison, it was found that PD-1+CD8+ T cells in the healthy individual express CD45RA but not CD27. On the contrary, PD-1+CD8+ T cells in the HBV HCC individual express CD27 but not CD45RA. This may lead us to the further investigate the function of PD-1+CD8 T cells and its phenotypes in different HBV associated disease stages. This information would be helpful in hypothesis generation and identification of rare populations at single-cell level. Therefore, SPADE analysis would be useful as a supplementary tool relating to conventional flow data analysis. It is proposed to combine the application of CITRUS and SPADE analysis along with the existing conventional flow data analysis to characterize of the expression pattern of inhibitory receptors on T cells. In the near future, we plan to apply it in data collected from tissues.

5.2.4 Visualizing high dimensional flow cytometric data to single cell level by viSNE algorithms

viSNE is an algorithm based program that reduces high dimensional flow cytometric data into 2-dimensional scatter plots while maintaining the structure of cells[192]. Its core algorithms were firstly developed in 2008 known as t - Distributed Stochastic Neighbor Embedding (t-SNE) in order to visualize multiple-parameters data in multiple fields[270]. From 2009 to 2014, its algorithms have been improved to preserve the local structure of data[271, 272]. With the development of mass cytometry, viSNE was applied to the identification of leukemia cancer cells in human cancerous bone marrow samples at single-cell level through CytoF[192]. The algorithms tested cells through several steps. 1: based on all the parameters in the experimental panel, viSNE calculates the fluorescent intensity of each marker on the cells; 2: then viSNE algorithms run a pair-wise study to compare the similarities of cells contained in the samples based on sole marker expression and co-expression of multiple markers; 3: next, viSNE algorithms rank all the cells obtained from the samples according to its expression of all the markers in the panel; 4: finally, visualization algorithms plot the entire population selected into a 2 dimensional scatter plot at single-cell level. Here, phenotypically similar cells would be aggregated into clouds in a viSNE map, while maintaining the “distance” of cells.

With its mechanism understood, we propose that viSNE may be an additional tool to reveal the heterogeneity of tumor infiltrating lymphocytes as the results from Chapter 3 suggested that inhibitory receptors on TILs demonstrate heterogeneous expression even from individuals with the same type of cancer

(wide range of PD-1 expression on TILs in a breast cancer cohort). In addition, apart from currently approved inhibitory pathway blockers such as Nivolumab and Pembrolizumab, additional inhibitory pathways including Tim-3, 2B4 and CD160 are under active analysis, some of which are in pre-clinical stage and some of which are in Phase I clinical trials[171]. Hence, visualization of its expression at single-cell level from human cancer tissues may reveal critical information that could accelerate the blockade investigation. Furthermore, visualizing high dimensional data from TILs when compared with that from non-cancerous benign lesions, may also identify the rare lymphocytic population that specifically resides in the tumor microenvironment, which may help us to precisely target the promising subsets of TILs for blockade immunotherapy in order to reinvigorate anti-tumor responses. Moreover, viSNE may be useful in the inhibitory pathway blockade clinical trials as one the focuses for researchers in PD-1/PD-L1blockade clinical trials is to elucidate the characteristics of TILs between PD-1 inhibitor non-responders and PD-1 inhibitor responders.

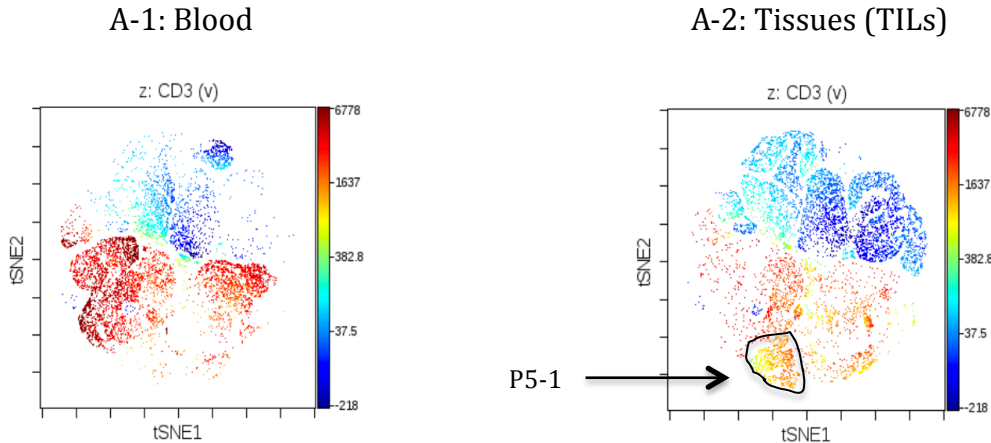
Table 5-2:

	Patient 1	Patient 2	Patient 3
Diagnosis	Triple negative breast cancer	Non-triple negative breast cancer	Breast fibroadenoma
Age	61	50	16
Sex	Female	Female	Female
TMN stage	III	I	None
PD-1 expression in PBMCs (%)	1.18% of CD8 T cells	4.07% of CD8 T cells	0.56% of CD8 T cells
	2.69% of CD4 T cells	5.84% of CD4 T cells	1.05% of CD4 T cells
PD-1 expression in tissues (%)	14.1% of CD8 TILs	32.1% of CD8 TILs	3.25% of CD8 TILs
	39.6% of CD4 TILs	22.4% of CD4 TILs	9.98% of CD4 TILs
PD-L1 expression in tissues	359.727 AFI	756.721 AFI	22.6 AFI

We optimized and applied viSNE algorithms to visualize and characterize the expression of inhibitory receptors on TILs from 2 selected individuals in a breast cancer cohort controlled with 1 breast adenofibroma individual at single-cell level. All 3 individuals have paired blood samples for comparison. Patient 1 has the poor prognosis pathological classification-triple negative breast cancer and Patient 1 is in TNM stage III with low PD-1 expression on CD8+ TILs (% of PD-1 on CD8+ TILs=14.1). Patient 2 is non-triple negative, TNM stage I breast cancer individual with moderate PD-1 expression of CD8+ TILs ((% of PD-1 on CD8+ TILs=32.1). Patient 3 is breast fibroadenoma individuals with low PD-1 expression of CD8+ FILs ((% of PD-1 on CD8+ TILs=3.25). Blood and tissue paired viSNE results of 3 individuals were illustrated in Figure 5-10.

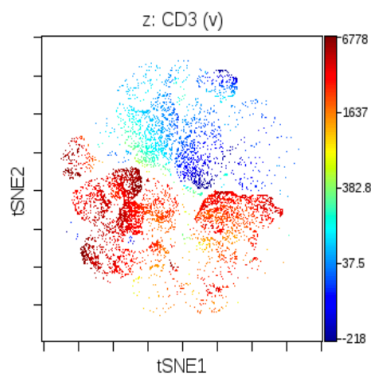
Figure 5-10: viSNE enables the identification of absent population at single-cell level in paired wise study

PATIENT 1-CD3

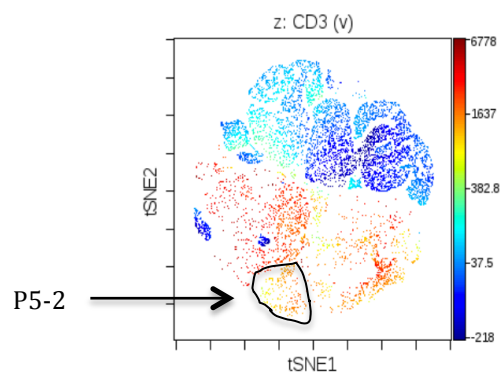


PATIENT 2-CD3

B-1: Blood

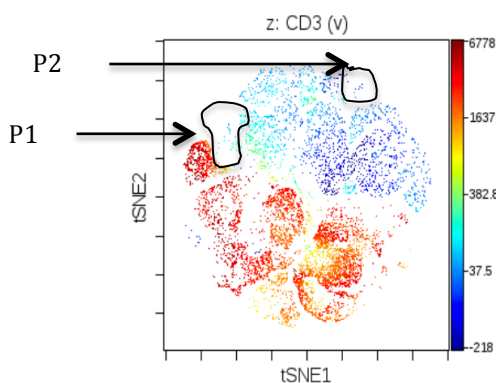


B-2: Tissues (TILs)



PATIENT 3-CD-3

C-1: Blood



C-2: Tissues (FILs)

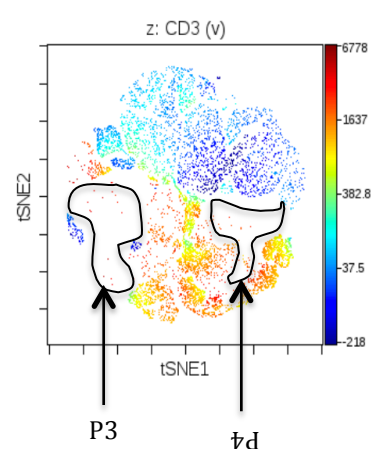


Figure 5-10: Dot plots of the viSNE maps from 3 blood-tissue paired (A to C) samples. Each dot in the map represents one single cell from samples. Median fluorescent expression of CD3 is depicted in color gradient manner. Each map is gated on population of singlets from “live cells” (negative population on live/dead staining). viSNE map consists of several large populations and a number of small separated subpopulations. These populations could be further characterized by the expression of other markers expression in the same panel. P1to P5 represents the population may be absent or present in the comparison between blood and paired tissues or between malignant samples and benign samples.

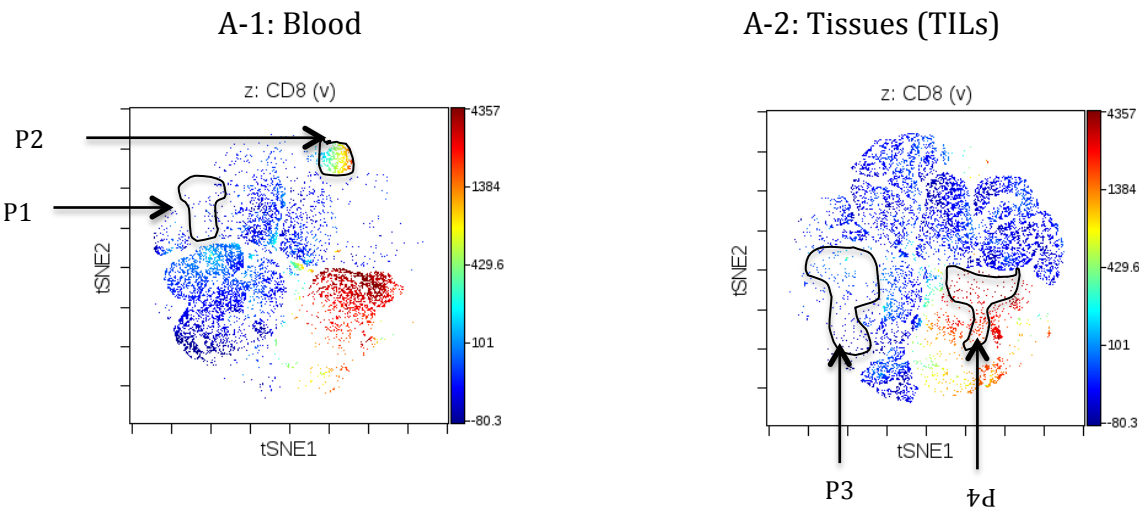
In Figure 5-10, viSNE maps enable us to identify the population (P1) that was absent in the blood but was present in the tissues. This was observed in all 3 patients, which indicates it may represent a tissue-resident population. In the comparison of population in the peripheral blood between 2 breast cancer individuals and 1 benign breast adenofibroma individual, the P2 population has dense aggregation of cells in the peripheral blood from 2 malignant patients but was absent in the benign breast lesion patient. This suggested that the P2 population in the blood may be specifically present in breast cancer individuals, although thorough studies should be conducted to confirm its phenotypes and function in a large cohort.

Furthermore, in the comparison of TILs from the 2 malignant breast cancer individuals and the 1 benign breast tissue, P3 and P4 subpopulations were only observed in cancerous tissues not in benign tissue. Since P3 and P4 all express CD3, it implies that those subpopulations resided in a tumor microenvironment and may be tumor infiltrated T cells that could have critical roles in T –cells mediated anti-cancer responses. In addition, in the comparison of 2 breast cancer individuals, one of the CD3+ aggregations has more cells (P5-1) in the triple negative breast cancer patient than that (P5-2) of early stage non-triple negative breast cancer patient although this subset is also present in benign breast lesions. Hence, it indicates this subpopulation of T cells may associate with the prognosis of breast cancer. Such discoveries in the results in Figure 5-10 are hardly perceived in conventional flow cytometric data analysis. Therefore, viSNE would be an useful and additional tool in analyzing

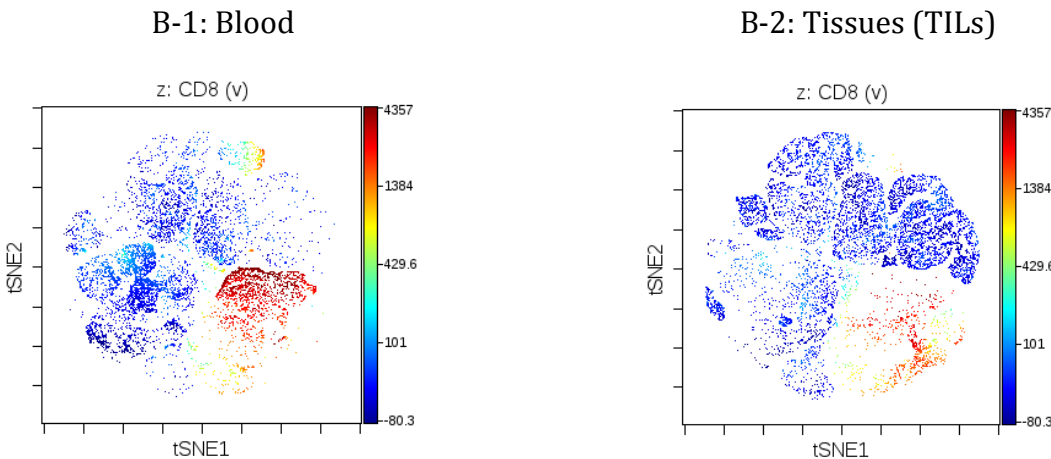
heterogeneous populations, such as TILs, to further develop the explanation from results in Chapter 3 where the diverse sole or combinatorial expression of inhibitory receptors on TILs was observed in 43 breast cancer individuals. Next, we investigated other markers expression on these 3 blood-tissue paired patients, to characterize the expression inhibitory receptors in each individual at single-cell measurement in Figure 5-14.

Figure 5-11: CD8 and CD4 expression in viSNE maps from 3 blood-tissue paired individuals

PATIENT 1-CD8

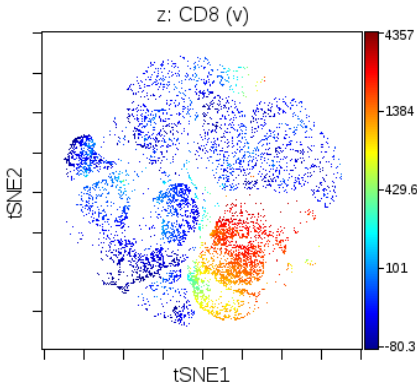


PATIENT 2-CD8

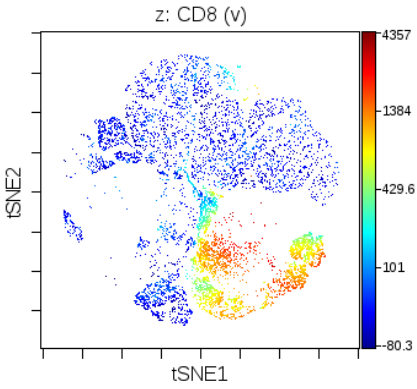


PATIENT 3-CD8

C-1: Blood

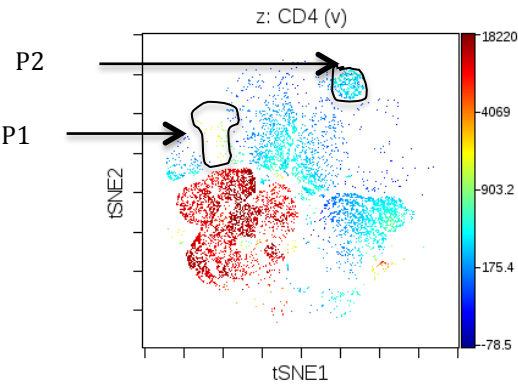


C-2: Tissues (FILs)

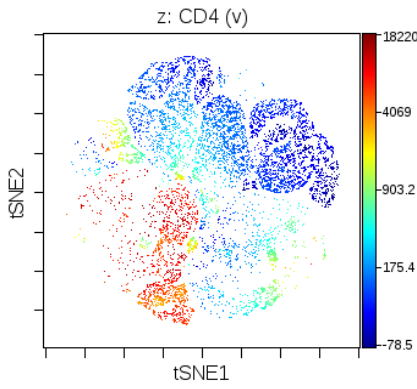


PATIENT 1-CD4

D-1: Blood

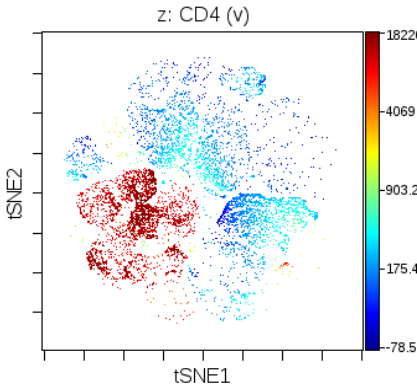


D-2: Tissues (TILs)

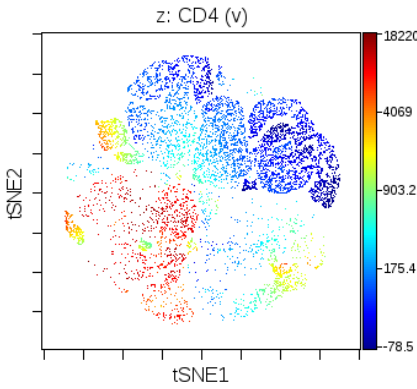


PATIENT 2-CD4

E-1: Blood



E-2: Tissues (TILs)



PATIENT 3-CD4

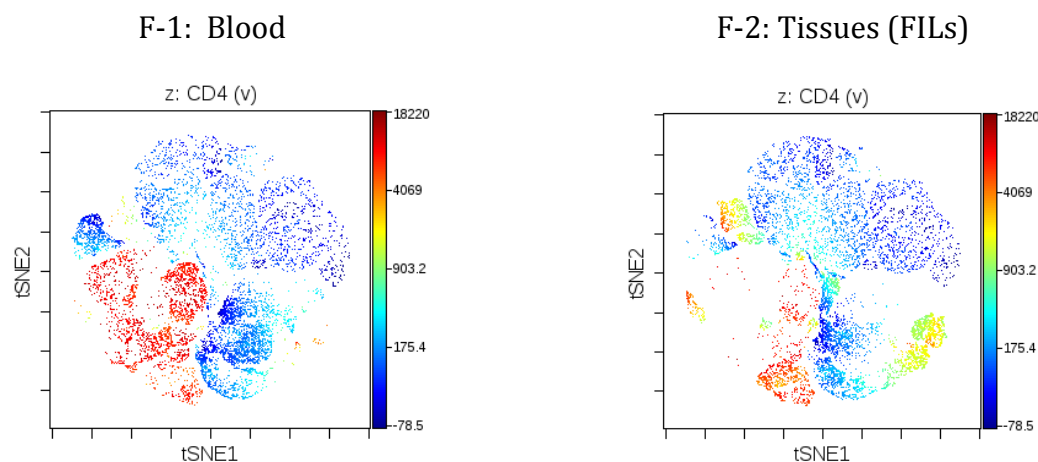


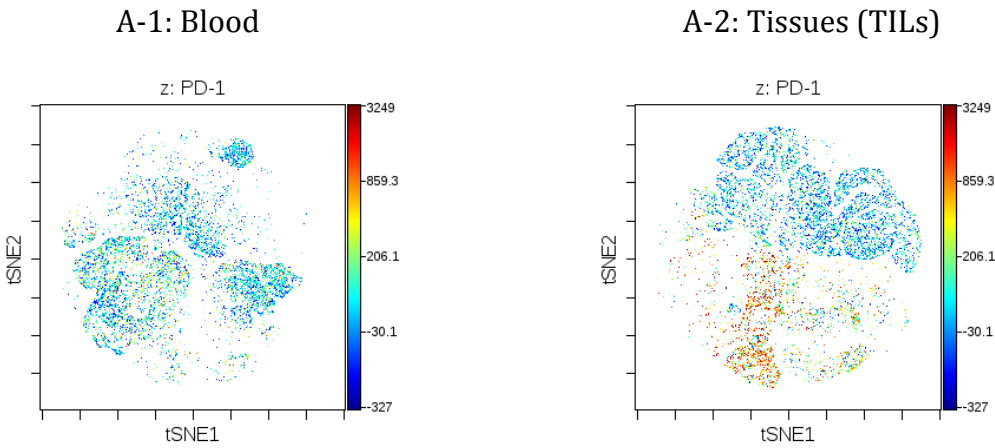
Figure 5-11: Dot plots of the viSNE maps from 3 blood-tissue paired (A to F) samples. Each dot in the map represents one single cell from samples. Median fluorescent expression of CD4 and CD8 is depicted in color gradient manner. A-C are CD8 expression viSNE maps and D-F are CD4 expression viSNE maps. Each map is gated on population of singlets from “live cells” (negative population on live/dead staining). viSNE map consists several large population and a number of small separated subpopulations that are non T cells. These populations could be further characterized by other markers expression in the same panel.

From viSNE results in Figure 5-11, we are able to further reveal the phenotypes of CD3⁺ subsets. In the abovementioned CD3⁺ subsets, P3 and P4 are the CD4⁺T cells subset and the CD8⁺ T cells subset respectively. P5 is also a subpopulation in CD4⁺ T cells. Hence, P1 may represent a subset of unconventional T cells, which may be NK T cells or delta/gamma T cells. Further characterization of this subset requires additional makers in the panel to distinguish it from other T cell subsets. In addition, P2 comprises CD3⁻ subsets but in the peripheral blood of all 3 individuals, a part of this subset has a low expression of CD8. In the comparison with paired tissue samples, either malignant or benign, the P2

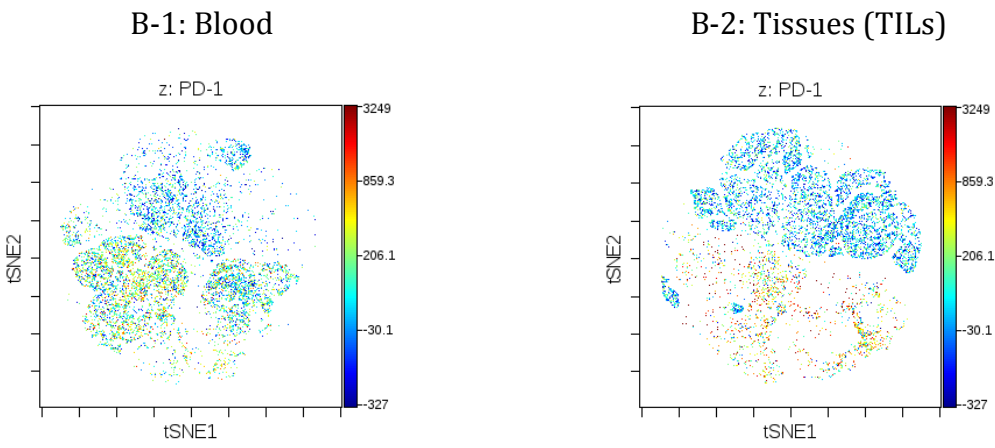
subset was significantly reduced in tissues. It suggested that such a subset may have diagnostic value rather than therapeutic value resulting from its absence in tumor microenvironment. In contrast, the P1 subset in Figure 5-10, has low frequencies in the periphery when compared with its paired tissue data. P1 subset is CD3- but has low expression of CD4 in both malignant and benign tissues. Our results have no indication of the P1 subset and it may require further characterization with additional markers. With the clear characterization of CD4 and CD8 T cells subsets, we should next determine the expression of inhibitory receptors on different lymphocytic subsets, which was illustrated in Figure 5-12. It may bring out more information of co-expression pattern of inhibitory receptors in other lymphocytic subsets that are crucial in the design of immunotherapy.

Figure 5-12: Characterizing the expression of inhibitory receptors in viSNE maps from 3 blood-tissue paired individuals

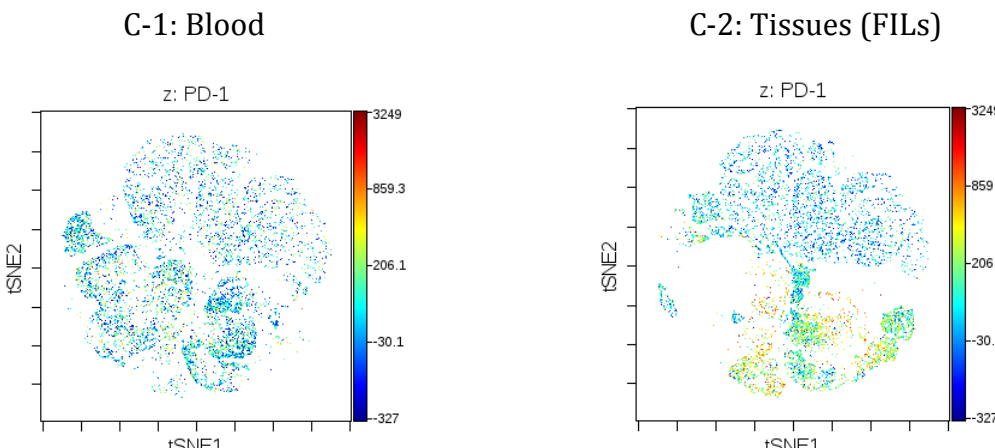
PATIENT 1-PD-1



PATIENT 2-PD-1

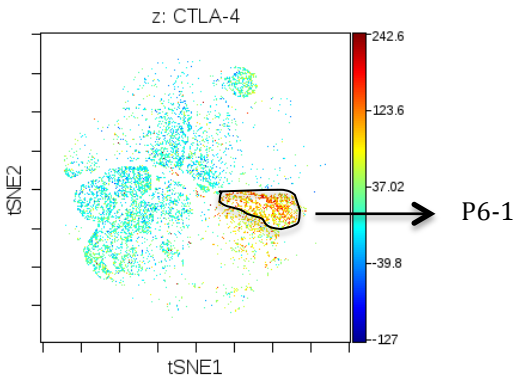


PATIENT 3-PD-1

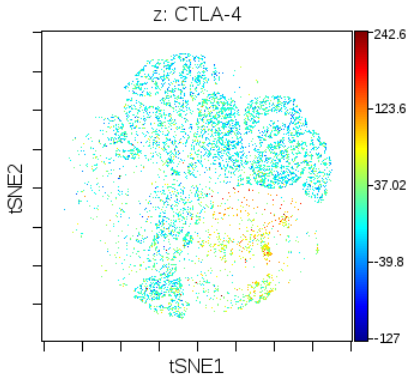


PATIENT 1-CTLA-4

D-1: Blood

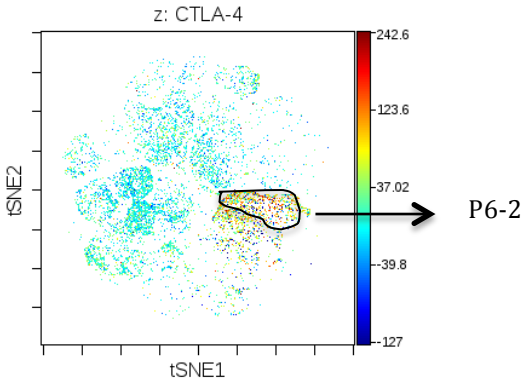


D-2: Tissues (TILs)

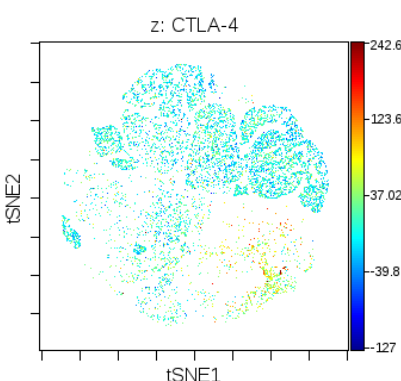


PATIENT 2-CTLA-4

E-1: Blood

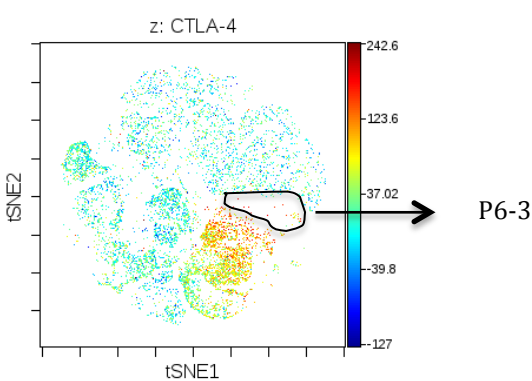


E-2: Tissues (TILs)

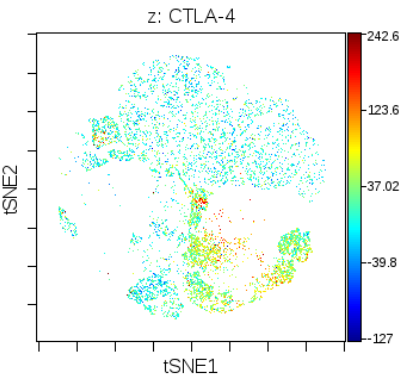


PATIENT 3-CTLA-4

E-1: Blood

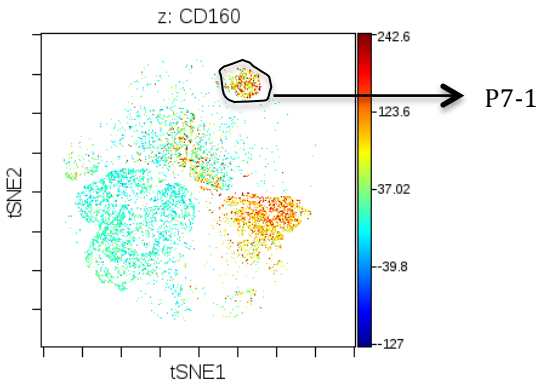


E-2: Tissues (FILs)

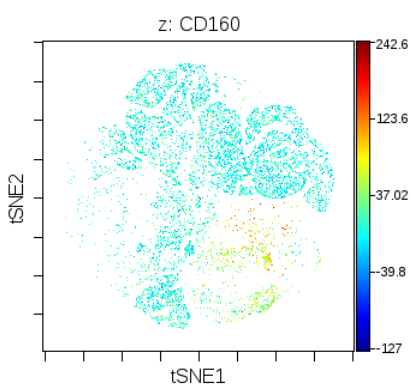


PATIENT 1-CD160

F-1: Blood

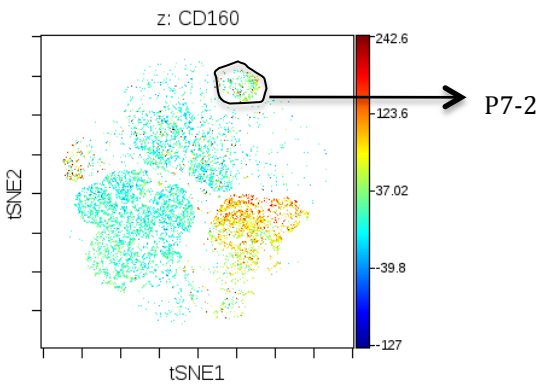


F-2: Tissues (TILs)

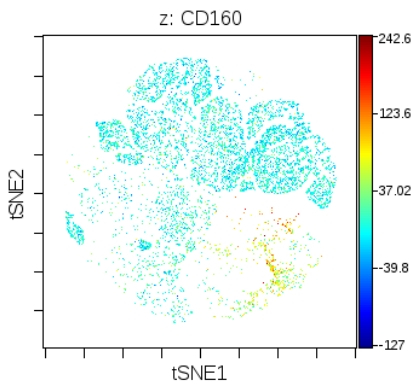


PATIENT 2-CD160

G-1: Blood

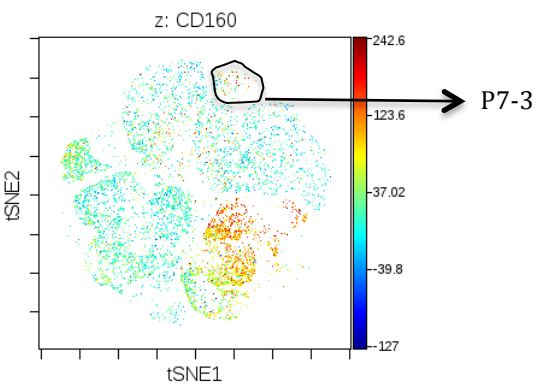


G-2: Tissues (TILs)

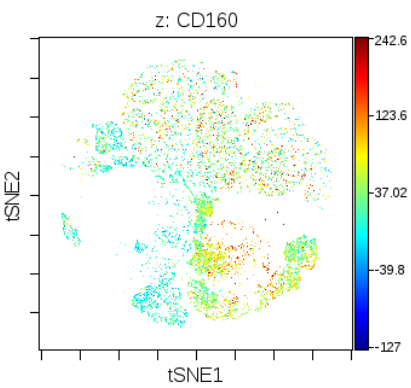


PATIENT 3-CD160

H-1: Blood

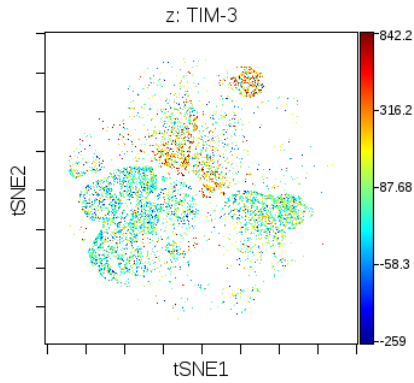


H-2: Tissues (FILs)

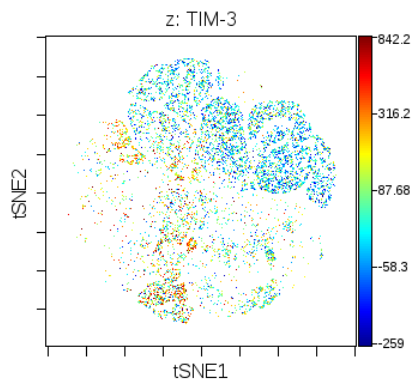


PATIENT 1-Tim-3

I-1: Blood

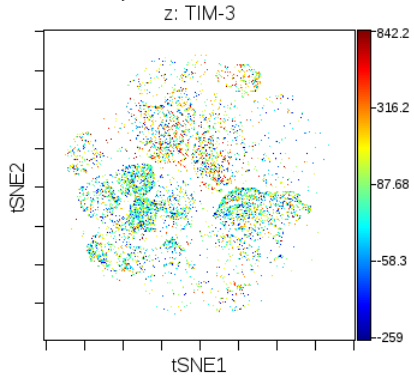


I-2: Tissues (TILs)

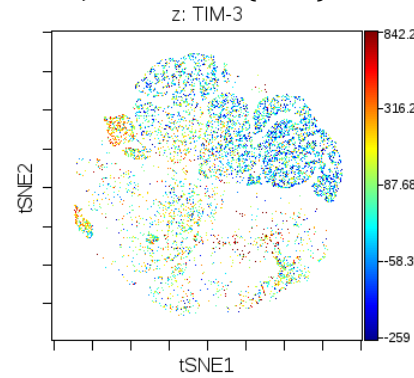


PATIENT 2-Tim-3

J-1: Blood

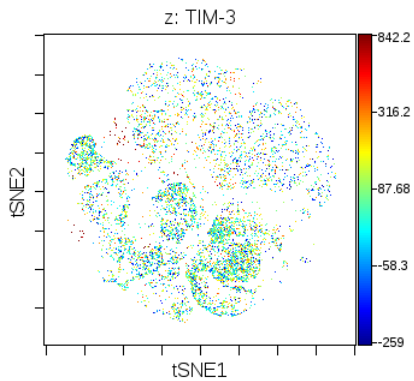


J-2: Tissues (TILs)

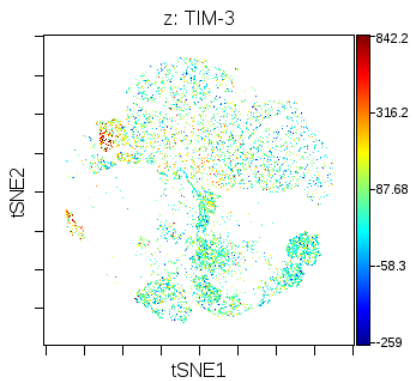


PATIENT 3-Tim-3

K-1: Blood

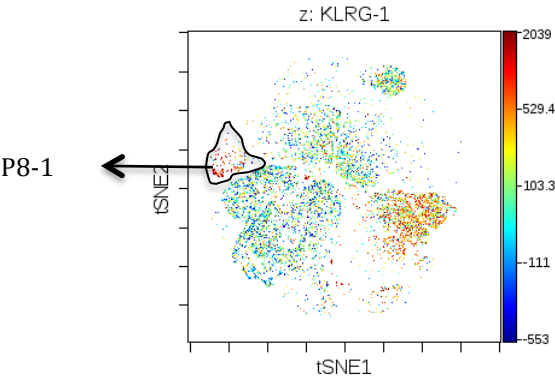


K-2: Tissues (FILs)

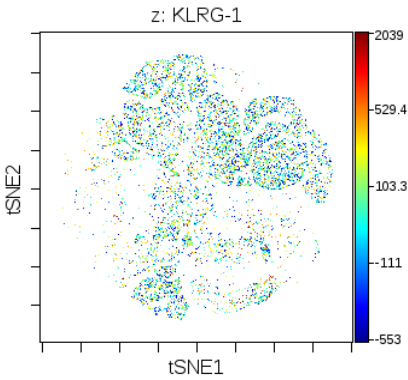


PATIENT 1-KLRG-1

L-1: Blood

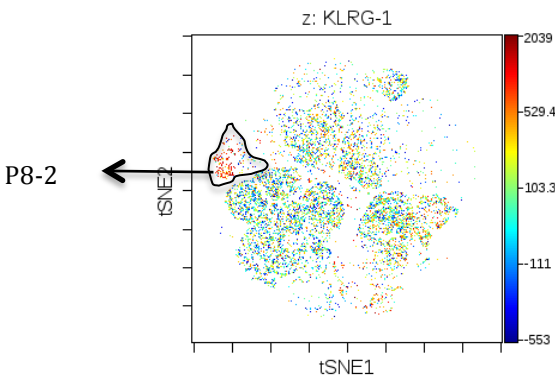


L-2: Tissues (TILs)

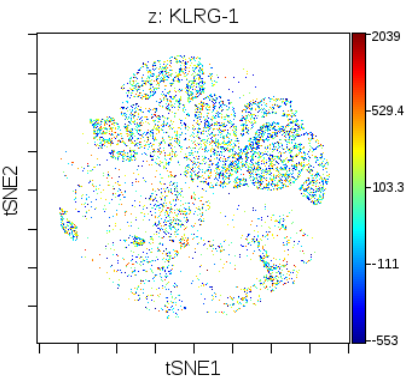


PATIENT 2-KLRG-1

M-1: Blood

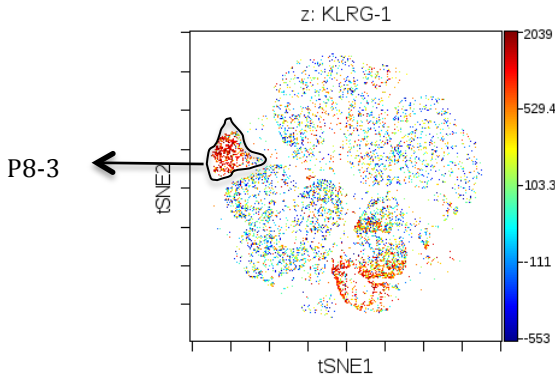


M-2: Tissues (TILs)

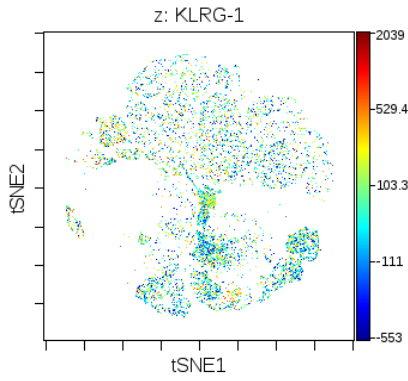


PATIENT 3-KLRG-1

N-1: Blood

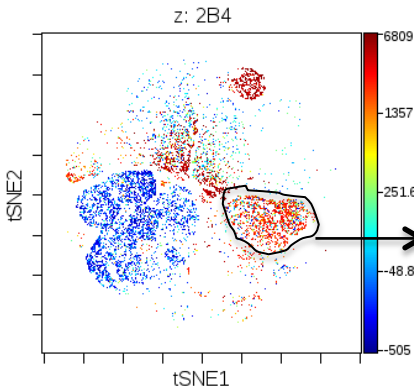


N-2: Tissues (TILs)

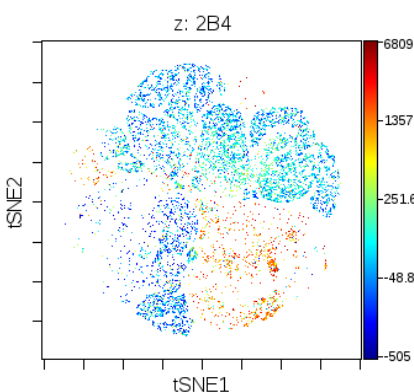


PATIENT 1-2B4

O-1: Blood

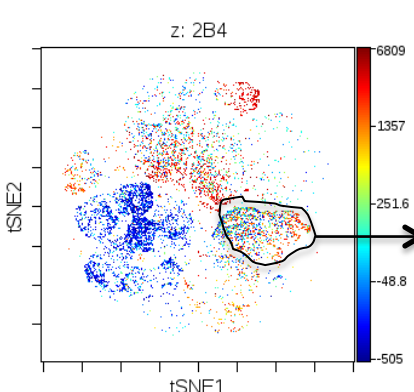


O-2: Tissues (TILs)

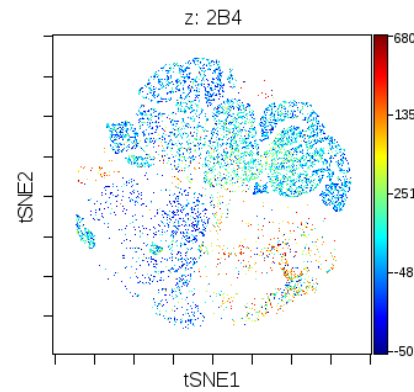


PATIENT 2-2B4

P-1: Blood

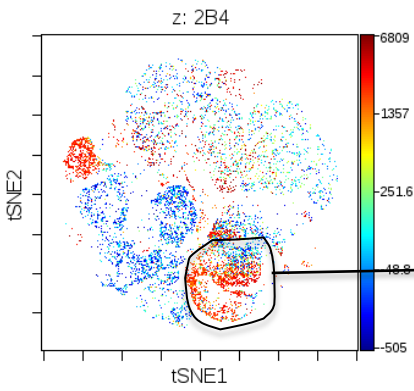


P-2: Tissues (TILs)

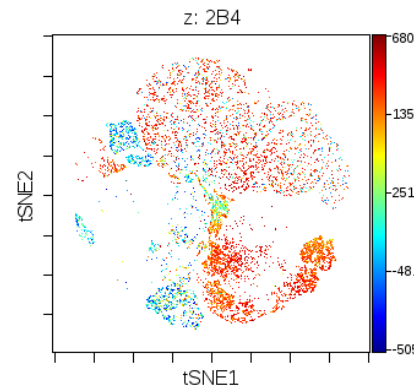


PATIENT 3-2B4

Q-1: Blood

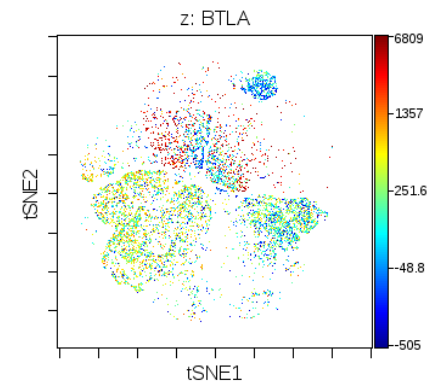


Q-2: Tissues (FILs)

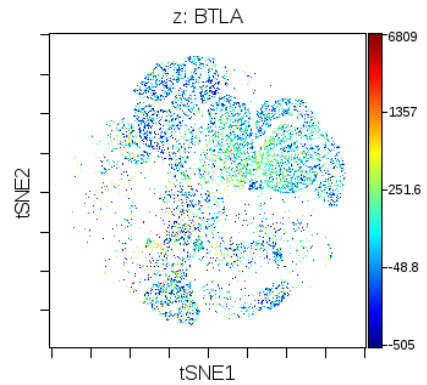


PATIENT 1-BTLA

R-1: Blood

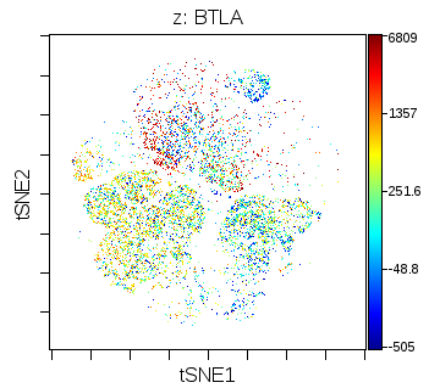


R-2: Tissues (TILs)

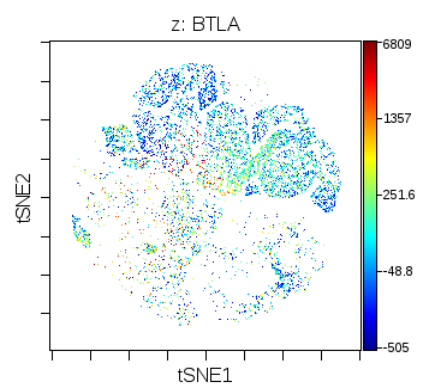


PATIENT 2-BTLA

S-1: Blood

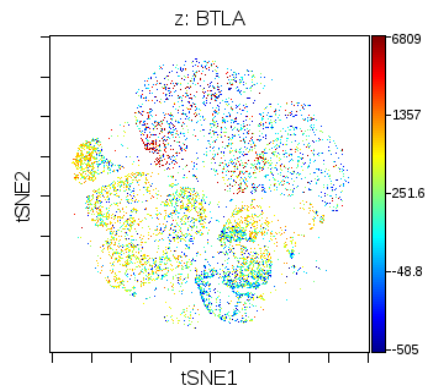


S-2: Tissues (TILs)



PATIENT 3-BTLA

T-1: Blood



T-2: Tissues (FILs)

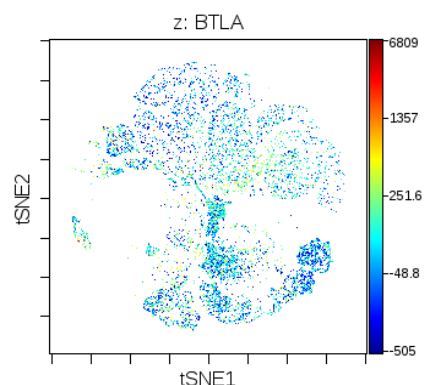


Figure 5-12: Dot plots of the viSNE maps from 3 blood-tissue paired samples. Each dot in the map represents one single cell from samples. Median fluorescent expression of **A-C)** PD-1, **D-E)** CTLA-4, **F-H)** CD160, **I-K)** Tim-3, **L-M)** KLRG-1, **O-Q)** 2B4, and **R-T)** BTLA is depicted in a color gradient. Each map gated on population of singlets from “live cells” (negative population on live/dead staining). The viSNE map enables expression analysis of inhibitory receptors in several large populations and a number of small separated subpopulations that are non T cells simultaneously.

In Figure 5-12 (A1-C1), viSNE maps indicated that, in the peripheral blood, no significantly differential expression pattern of PD-1 was observed in all lymphocytic subsets from the 3 individuals. PD-1 has a relatively low expression even in the 2 breast cancer individuals. However, PD-1 had lower expression on T cells benign breast tissues (Figure 5-12 C2) but mild expression in both CD4 and CD8 on TILs in breast cancer patients (Figure 5-12 B2).

Of note, (Figure 5-12 A2-B2) in the comparison of PD-1 expression on TILs, between the advanced stage, triple negative breast cancer individual (Patient 1) and the early stage non-triple negative breast cancer individual (Patient 2), it was evident that PD-1 has lower expression on CD8+ TILs in Patient1 than that of Patient 2. In addition, most of the PD-1 expression aggregated in CD4+ TILs subset. Higher expression of PD-1 in the CD4 TILs may contribute to immunosuppression in the tumor microenvironment, especially that from Tregs. However Patient 2, PD-1 has relatively scattered expression on both CD4 and CD8 TILs. Currently PD-1 inhibitors have been applied in triple negative breast cancer treatment. The implication is that PD-1 inhibitors may also diminish immunosuppressive influences that tumor infiltrated Tregs brought to the tumor microenvironment but more in vitro experiments would be essential to conclude.

From the benefits PD-1 inhibitors achieved, we may have implications of the characteristics of perspective inhibitory pathway for future blockades. 1: as a prerequisite, it is functionally proved to elicit inhibitory signaling resulting to T-cell exhaustion. 2: it has relatively low expression in the peripheral blood but high expression on TILs. 3: blockade of inhibitory pathway improves T-cells mediated anti-cancer responses. 4: its expression is mainly on T cells but not other subsets. 5: its application has manageable adverse effects. viSNE maps may enable us to examine those additional inhibitory receptors by following above rules in individual patient at single-cell level.

CTLA-4 inhibitor is the first blockade medication approved by FDA. It has noteworthy benefits in treating advanced melanoma. However, different from PD-1 inhibitors, CTLA-4 inhibitor demonstrated severe, high-grade, immune-related adverse effects that limits its application in other cancers[78]. In Figure 5-11 D-F, it was found that CTLA-4 expression was predominately clustered in the CD8 T cells in both peripheral blood and tissues. Interestingly, the presence of a subset with relatively high expression of CTLA-4 in the 2 breast cancer patients (P6-1 to 2) was absence in Patient 3 (P6-3), the benign breast adenofibroma patient. But the general expression of CTLA-4 is low which is indicated from the median fluorescence in viSNE maps.

In Figure 5-12 F-H, it was shown that CD160 has similar expression pattern, as it was observed in CTLA-4 viSNE maps in both the peripheral blood and tissues from 3 individuals. Population (P-6) observed in CTLA-4 viSNE maps co-express

CD160 in the blood from breast cancer individuals but this population was absent in Patient 3. Notably, population P7 in CD160 viSNE maps demonstrated high expression of CD160 only in Patient 1's peripheral blood, the triple negative breast cancer individual (P-7-1) when compared with its expression of P7 in peripheral blood from Patient 2 and Patient 3 (P7-2 to 3). This population (P7-1) in Patient 1 also co-express high expression of 2B4 and Tim-3 and dim expression of KLRG-1. It is phenotypically quite a distinguishable population in the blood in Patient 1 when compared with Patient 2 and Patient 3. As Patient 1 is the only triple negative breast cancer individual in our cohort, we could not further apply viSNE to check if this subset is specifically observable in triple negative breast cancer individuals but not in non-triple negative cancer individuals. As this population is only present in the blood, if it is only observable in triple negative breast cancer patients, it would have the potential to be a biomarker.

In Figure 5-12 I-K, it is found that in the peripheral blood of 3 individuals, Tim-3 has lower expression on the T cells but relatively higher expression in non-T cells subsets. However, in the tissues, Tim-3 has higher expression in T cells from 2 cancer patients (Figure 5-12 I2 and J2) while maintaining low expression on T cells from breast adenofibroma patient (Figure 5-12 K-2). Combining results obtained in Chapter 3 that Tim-3 was selectively upregulated on T cells from malignant breast tissues but not benign breast tissues, it suggested that in clinical trials of Tim-3 pathway blockade, local inject of Tim-3 inhibitor may result in less adverse effects than that seen in systemic infusion.

In Figure 5-12 L-M, it was shown that KLRG-1 has higher expression in CD8 T cells in peripheral blood from all 3 individuals. It also has a higher expression in CD3+ but not CD4 or CD8 T cells (P8). This subset also co-expresses 2B4. Its phenotype and function requires further investigation. In tissues, KLRG-1 expression was mainly in CD8 T cells from breast cancer individuals (Patient 1 and 2) while maintaining low expression in benign CD8 T cells. Overall its expression on CD8 TILs is generally low in 2 breast cancer individuals. This indicates that KLRG-1 may have the potential in the development of blockade inhibitors.

In Figure 5-12 O-Q, it was found that 2B4 has higher expression in CD8 T cells rather than CD4 T cells in the peripheral blood, although its expression could also be seen in non-T cell clusters in blood from all 3 individuals. Interestingly, in the peripheral, 2B4+ CD8 T cells (P9-1, P9-2) from 2 malignant breast cancer individuals (Patient 1 and 2) clustered in distinct viSNE clouds when compared with (P9-3) that in benign breast disease individual (Patient 3). This may indicate the 2B4+ CD8 T cells in peripheral blood in the breast cancer may have distinctive phenotype and function when compared with that in the non-breast cancer individual. And this CD8 T cell subset may have therapeutic potential for further analysis. In addition, 2B4 has higher expression on CD8 T cells in both malignant and benign tissues. This may suggest that upregulation of 2B4 in CD8 T cells benign lesions may act in a role of normal physiological function.

In Figure 5-12 R-T, it was shown that BTLA has relatively low expression on T cells in peripheral blood and tissues from all 3 individuals. Our interpretation

along with other studies regarding to BTLA is that it is rather a T-cell differentiation marker that highly expressed in naïve T cell phenotype while losing its expression along with T-cells differentiation. Hence, it has less value for the development of blockade immunotherapy.

In conclusion, viSNE enables us to perform paired wise expression analysis of multiple inhibitory receptors on different subsets from cancer patients. It facilitates the identification and visualization of expression pattern of inhibitory receptors on different subsets simultaneously at single-cell level. It reduces high dimensionality data into 2 dimensional viSNE map while maintaining “cell-cell distance”. It is similar to SPADE analysis but focusing on single-cell display and the structure of cells whereas SPADE is superior in clustering rare population. Therefore, the combination of SPADE and viSNE analysis would profoundly accelerate and enhance expression analysis of interesting individuals at single-cell level from multi-parameters data. More importantly additional but exclusive information yielded from SPADE and viSNE analysis could fill the gap in conventional analysis method.

5.2.5 Discussion

In this chapter, we firstly engaged in the characterization of the expression of inhibitory receptors on peripheral T cells from healthy donors, CHB individuals, HBV associated cirrhosis individuals and HBV associated HCC individuals. However, due to the heterogeneity of T-cell populations, we were unable to identify the differential expression of inhibitory receptors on T cell from individuals in 4 abovementioned experimental groups. Even no differential expression of inhibitory receptors on peripheral T cells between healthy donors and disease individuals was observed. This led us to the application of next-generation bioinformatics approaches in analyzing high dimensional flow data.

3 types of algorithms were applied in our studies in order to answer different research questions. Although CITRUS, SPADE and viSNE are all programming models designed for mass cytometry, we successfully apply it in analyzing data acquired from fluorescent cytometry. Indeed, with the development of advanced technologies in cytometry, we are capable of performing mutichromatic staining at a single cell up to 14 different markers simultaneously in fluorescent flow cytometry. In mass cytometry, it could be extended to over 40 markers. Critical information could be squeezed from those high dimensional data. However, conventional supervised analysis by defining subsets and quantifying functional markers expression on subsets respectively slows the progression of limits the identification of rare population. Therefore, reducing high dimensionality and automated identification of subsets would become the key focuses in the future of quantitative and qualitative cellular assays.

Current fluorescent flow cytometry and advanced mass cytometry are all useful tools in cellular analysis with different strength. In the Table 5-3, I summarized the characteristics of both techniques. Overall, fluorescent cytometry has more powerful fluidic system enabling high-speed acquisition with high sensitivity but compensation is an obstacle for most of the users. In addition, machine's performance and quality control are of prominent importance. However, mass cytometry is superior in compensation, as it does not require compensation in metal isotopes detection system. However, its acquisition efficiency is a shortage that limits the application of large-scale data collection in short time. In all, both techniques enable us to perform high dimensional data collection in order to answer critical research questions.

Table 5-3: Comparison of fluorescent cytometry and mass cytometry

	Fluorescent cytometry	Mass cytometry
Detection mechanism	Fluorescence	Metal isotopes
Compensation	Yes	No
Sorting	Yes	No
Sampling efficiency	>95%	<30-50%
Acquisition speed	Up to 25,000 per second	500-1,000/second
Max parameters	Beginner: 4-8	40-50
	Intermediate: 8-12	
	Advance: 12-16	

In our studies, we applied CITRUS analysis to further reveal potentially differential expression of inhibitory receptors on lymphocytic subsets among different HBV associated disease stages. The hierarchical clustering and predictive modeling in CITRUS enables the identification of specific subsets that

were masked in bulk CD8 T cells and NK cells, from which differential sole expression or co-expression pattern of inhibitory receptors could be observed among different experimental groups. These subsets may play important roles in the pathogenesis and progression of chronic HBV infection and HBV associated carcinogenesis. Further investigation includes the functional studies of those subsets.

Furthermore, application of SPADE analysis and viSNE analysis facilitates us to visualize high dimensional data in 2 dimensional plots at single-cell level. Both algorithms allow us to simultaneously quantify the expression of inhibitory receptors on different subsets. But SPADE focuses the on identification of rare population and dynamic change of immune-signatures in lymphocytes. In the mean while, viSNE concentrates on the maintenance of cell-cell distance in single-cell level and has great potential in routine clinical examinations or biomarker screening. The comparison of SPADE and viSNE algorithms was summarized in recent study and listed below in table 5-4 [269].

In all, based on the abovementioned benefits 3 algorithm based analysis brought, we would apply it integrally for the analysis of the expression of inhibitory receptors to reveal the heterogeneity of tumor infiltrating T cells in order to obtain additional information in the design of blockade immunotherapies.

Table 5-4: Comparison of SPADE and viSNE algorithms

Feature	SPADE	viSNE
Implementation features		
Required input parameters	Set of markers; number of cell clusters	Set of markers
Input data type	FCS file	FCS file
Data transformation on input data	Any	Arcsinh
Data down-sampling	Density dependent	Random
Sensitive to down-sampling	Yes	Yes
Single-cell visualization	Only on a small data set that does not require the generation of cell clusters	Yes, after random down-sampling
Software implementation	MATLAB, R, Cytoscape	MATLAB/Cyt
Maximum sample size for visualization after down-sampling	50,000 cells	30,000 cells
Run time for 50,000 cells	Approximately 5 min	Approximately 15 min
Visualization	2D tree plot	2D or 3D dot plot
Utilization features		
Subpopulation identification	Cell clustering for building 2D tree, then manual interpretation of branches for population identification	No clustering; manual interpretation of visually distinct clusters for population identification
Comparison of multiple data sets	Yes	Yes
Automatic projection of new cells	No	No
Infer cellular hierarchy	Yes	No
Stable local data structure	Yes	Yes
Suitable for rare population identification	Yes	Yes

Table was adapted from Benedict Anchang et al; Nature Protocol June 2016

6: Final discussion and future direction

6.1 Multiple roles of inhibitory receptors

In this thesis, we have characterised the expression of 8 inhibitory receptors, PD-1, CTLA-4, Tim-3, KLRG-1, 2B4, TIGIT, CD160 and BTLA, on T cells for comparative analysis in multiple cancers. Some of them, such as PD-1 or CTLA-4, have been already developed into medications for cancer immunotherapies and delivered promising benefits in advanced cancer individuals[52, 54, 64, 70, 76, 79, 80, 102, 104, 195, 250]. Its application could also extend to more types of solid cancer [73]. Studies in this thesis broadened our interpretation of inhibitory receptors' role in chronic virus infection and cancers. Initially, the concept of T-cell exhaustion was raised to define dysfunctional T cells, a hyporesponsiveness state of T cells accompanied with poor cytokines production, activation of apoptosis signalling and upregulation of multiple inhibitory receptors. This has been observed on T cells in chronic HBV infection, chronic HCV infection, and chronic HIV infection in both animal experiments and human studies[199, 200, 202, 223, 273]. Since then, the upregulation of multiple inhibitory receptors on T cells in chronic virus infection and cancers have been proposed to define T-cell exhaustion, which indicates T cells with higher expression of inhibitory receptors are exhausted T cells. However, with more studies investigating the expression of inhibitory receptor on T cells, good evidence has been found that the expression and up-regulation of inhibitory receptors can be found on non-impaired T cells.

The up-regulation of inhibitory receptors on T cells is also an activation marker. In healthy individuals, it was found that upon activation of CD8 T cells in peripheral blood, multiple inhibitory receptors were significantly up-regulated. However, no major impairment of cytokine production in those inhibitory receptors-positive CD8 T cells was observed[214]. It suggests that the up-regulation of inhibitory receptors on peripheral T cells is not related to T-cell exhaustion but associated with T-cell activation in healthy individuals. This was not only observed in peripheral blood but also in lymph nodes in healthy individuals[214].

Moreover, the up-regulation and expression of inhibitory receptors on T cells is also tightly linked with T-cell differentiation. In healthy individuals, several inhibitory receptors have differential expression on different differentiated stages of T cells. For instance, BTLA has expression on more than 80% of naïve T cells but only 40% of terminally differentiated T cells[201]. In the meantime, most of the inhibitory receptors have higher expression in intermediate and advanced differentiated stages of T cells but lower expression in naïve differentiated T cells[206]. Those results observed in healthy individuals together with the expression levels of inhibitory receptors on T cells in chronic virus infection or cancer, indicated that the expression of inhibitory receptors is also tightly associated with T-cell differentiation.

In all, the expression and upregulation of inhibitory receptors on T cells cannot mark T-cell exhaustion in chronic infection or cancer, but also link with T-cell activation and differentiation. This implies that in our future studies, we would

integrate the cytokine production profiling of T cells with the expression patterns of multiple inhibitory receptors on T cells in order to identify the optimal strategies to reinvigorate dysfunctional T cells.

6.2 Tailored regimens for blockade immunotherapies

The tumor microenvironment is a complex property that harvests magnificent immunosuppressive effects to anti-cancer responses[207, 274]. It constitutes a unique structure with mesenchymal cells, stromal cells and endothelial cells to physically excluding immune cells into tumors[275, 276]. In addition, the tumor microenvironment involves tumor progression and metastasis as well as resistance to anti-cancer responses[277]. Hence, the tumor microenvironment has been thought as one of the key factors when considering treating solid tumors.

Different cancers comprise different tumor microenvironments resulting in differential prognosis. In order to maximize the benefits blockade immunotherapies brought to advanced cancer individuals, in this thesis, we investigated the distinctive expression of inhibitory receptors on TILs from 7 types of cancer. This study aims to identify the tailored regimens of blockade immunotherapies for different cancer individuals and we did find the differential expression of several sole inhibitory receptors on TILs in different tumor microenvironments. For instance, Tim-3 preferentially expressed on TILs from “digestive tract” cancers such as oesophageal cancer, gastric cancer and colorectal cancer. Furthermore, we have also characterized the combinatorial

expression patterns by multiple inhibitory receptors from 7 types of cancer. It was shown that HBV HCC has different dominant phenotype of CD8 TILs when compared with that in other cancers. In addition, 6 types of cancer share the same dominant phenotype of CD8 TILs but different sub-dominant phenotypes of CD8 TILs. Although further functional experiments need to be conducted, it indicates that different regimens could be determined for different types of cancer, when planning the blockade immunotherapies. This approach may allow more advanced cancer individuals to benefit from inhibitory pathway blockades. In addition, characterizing the co-expression patterns of inhibitory receptors on TILs at individuals level may also provide useful information in predicting who may have responses to the blockade.

In all, targeting tumor microenvironments in different types of cancer and at individual level may help us to better understand anti-cancer immunity, which plays an important role in the control of tumor progression and dissemination. Sole and combinatorial expression analysis of multiple inhibitory receptors on TILs may provide the rationale for the next generation of the blockade immunotherapies.

6.3 Identification of biomarkers for blockade immunotherapies

Since the approval of CTLA-4 and PD-1 inhibitors, numerous efforts have been spent in identifying the reliable biomarkers that could guide clinicians to distinguish who may respond to the inhibitory pathway blockades[216, 257]. Ideally, the prospective biomarkers could accurately predict who would be responders with high specificity and sensitivity. However, so far, in the practice of ipilimumab, the CTLA-4 inhibitor, in treating advanced melanoma individuals, no biomarkers were proposed or identified. In the use of PD-1 inhibitor, PD-L1 has been proposed as a potential biomarker to PD-1 inhibitory pathway blockade. However, the results revealed from more clinical trials in treating advanced melanoma individuals, on the one hand, that 30-40% of PD-L1+ advanced melanoma individuals demonstrated overall responses[75]. In other advanced cancers, such as non-small cell lung cancer, renal cancer, or triple negative breast cancer the ORR is lower than that seen in advanced melanoma[52, 79, 80, 278]. This means at least 60% of PD-L1+ advanced cancer individuals had no responses to PD-1 inhibitors. On the other hand, in a Phase III clinical trial, it was shown that 18% of PD-L1- advanced melanoma individuals also demonstrated responses to PD-1 inhibitors. Combining those results together, it is now controversial to use PD-L1 as a biomarker for blockade immunotherapies. Our interpretation is that PD-L1 may have different values as a biomarker in different cancers. Hence, it is necessary to determine PD-L1's value in each cancer type. Although we haven't had any clinical trial cancer individuals recruited in our cohort, we have observed the positive correlation between the expression of PD-1 on TILs and the expression of PD-L1 in non-

triple negative breast cancer individuals. It may indicate that PD-L1 may have the potential to become a biomarker in treating advanced non-triple negative breast cancer individuals.

Furthermore, in the efforts to determine whether there are correlations in 2 additional axis, between KLRG-1 and e-cadherin; Tim-3 and galectin-9, we did not observe either positive or negative correlations in 2 paired inhibitory receptor-ligand axis. This suggests that future work should involve the quantification of other ligands to evaluate which ligand has the potential to be the biomarker in the specific inhibitory pathway.

The biomarker has the pivotal role in the guidance of inhibitory pathway blockade to selectively distinguish responders from non-responders. Currently PD-L1 has value as a biomarker but needs to be evaluated in different types of cancer. Additional biomarkers require thorough investigation in both in vitro and in vivo experiments for each inhibitory pathway.

6.4 Adaptive resistance from blockade immunotherapies

From Phase I clinical trial to the approval of PD-1 inhibitors, Nivolumab or Pembrolizumab, use to treat advanced melanoma individuals, and non-small cell lung cancer individuals, it has been shown that the PD-1 blockade can restore

the anti-cancer T-cell responses with sustainable effects. However, after monitoring for several years, some of those advanced cancer individuals demonstrated disease progression and the PD-1 inhibitors no longer effective in eliciting anti-cancer responses[279]. Attentions should be paid to the PD-1 inhibitor adaptive resistance and the clinical strategies addressing adaptive resistance should be conceived accordingly.

Despite investigating the mechanisms of adaptive resistance established from the use of pathway inhibitors, the expression patterns of inhibitory receptors on TILs in the progressed tumors may have indications of alternative blockades that may prolong the anti-cancer responses. Adaptive resistance of PD-1 inhibitor resulted in it failing to control tumors. It has been reported that in lung cancer individuals who had adaptive resistance to PD-1 inhibitor, Tim-3 was upregulated on the PD-1 blocking antibody bound TILs[123]. These findings may suggest the alternative blockade, such as the Tim-3 pathway, may sustain the anti-cancer responses in PD-1 inhibitor resistant individuals. Our combinatorial expression analysis of inhibitory receptors on TILs may be useful in identifying the possible inhibitory pathways to block in controlling disease progression and in maintaining anti-cancer responses.

To date, the blockade immunotherapies have significantly prolonged the overall survival of advanced cancer individuals. This battle to find and apply new therapies in this area has just started and the aim is to maximize its benefits for those who are confronted terminal cancer illness.

6.5 Future plans

In the near future, this project aims to further develop as outlined below:

1: Recruiting more individuals into the study who have specific types of cancer, such as renal cancer individuals, thyroid cancer individuals and renal cancer individuals to strengthen current conclusions

2: Conducting functional assays on dominant phenotype of CD8 TILs in multiple cancers to characterize the degrees of T-cell exhaustion

3: Performing blockade experiments on dominant phenotype of CD8 TILs in multiple cancers to evaluate the functional restoration

4: Characterizing the gene expression profiling of dominant CD8 TILs in multiple cancers

7 Appendix:

Appendix-1:

Oxford Tropical Research Ethics Committee

University of Oxford
Research Services, University Offices
Wellington Square, Oxford OX1 2JD
Tel. +44 (0)1865 (2)82106
E-mail: oxtrecrec@admin.ox.ac.uk



Professor Tao Dong
MRC Human Immunology Unit
Weatherall Institute of Molecular Medicine
John Radcliffe Hospital
Oxford
OX3 9DS

11 August 2016

— Dear Professor Dong,

Full Title of Study: International cooperation between China and UK on Immunological and genetic association of virus/pathogen associated cancer development

OxTREC Reference: 587-16

Thank you for your email of the 9 August 2016, and for your minimal risk application form. Other documents reviewed were:

Documents:	Version:	Date:
Protocol	V8.0	08/08/16
Consent form	V2.0	
Xinjiang ethics approval		

The OxTREC executive team reviewed the above application on the 11 August 2016 and gave approval for this study.

Approval is given for the first five years and is subject to receiving the local ethical approval (if this approval has not yet been received).

Any subsequent changes to the application must be submitted to the Committee as an Amendment. This should include a letter to give the reasons for the proposed modifications and all revised documents with changes tracked.

Please ensure that you submit a completed Annual Report form on every anniversary of this approval and a final End of Study Report. The relevant forms can be found on the OxTREC website: <http://www.tropicalmedicine.ox.ac.uk/oxtrecrec>.

Yours sincerely

A handwritten signature in black ink, appearing to read "Mary Warrell".

Dr Mary Warrell
OxTREC Chairman

Direct Line Tel: +44 (0)1865 (2)82106
Email: oxtrecrec@admin.ox.ac.uk
Web: www.admin.ox.ac.uk/rso/

Appendix-2:

Oxford Tropical Research Ethics Committee

University of Oxford
Joint Research Office, Block 60
Churchill Hospital, Oxford OX3 7LJT
Tel. +44 (0) 1865 (5)72228, fax +44 (0) 1865 (5)72224
E-mail: fiona.goulthorp@admin.ox.ac.uk



Dr T Dong
MRC Human Immunology Unit
WIMM
John Radcliffe Hospital
Oxford
OX3 9DS

6th December 2011

Dear Dr Dong

– **Full Title of Study:** Immune-correlates of Hepatitis B Virus associated liver disease and Hepatocellular Carcinoma in China - A retrospective study

OXTREC Reference: 60-11

Thank you for your email 1st December 2011, in which you have responded to the committee's request for further clarification and amendments to protocol, now version 7.0, dated 1.12.2011.

We have reviewed the Oxford-based part of this research, which is a laboratory based study of samples obtained with informed consent in a larger project in You'an, China. As the Oxford-based study has minimal risks, I can, and do give OXTREC's approval by Chairman's action.

OXTREC are grateful for annual and end of study reports for this study.

Yours sincerely,

A handwritten signature in cursive script that reads 'Richard Mayon-White'.

Dr Richard Mayon-White

OXTREC Chair

Direct Line Tel: +44 (0)1865 (5)72224
Fax: +44 (0)1865 (5)72228 Email: fiona.goulthorp@admin.ox.ac.uk Web: www.admin.ox.ac.uk/rso/

Appendix-3:



Health Research Authority

London - City & East Research Ethics Committee

Bristol Research Ethics Committee Centre
Whitefriars
Level 3, Block B
Lewins Mead
Bristol
BS1 2NT

Telephone: 02071048033/53

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

07 October 2016

Professor Tao Dong
Professor of Immunology, Director of CAMS/Oxford Centre for translational Immunology, Nuffield
Department of Medicine, Oxford University
MRC Human Immunology Unit
WIMM/JR Hospital OX3 9DS

Dear Professor Dong

Study title: Immunological and genetic association of virus/pathogen linked with cancer development
REC reference: 16/LO/1607
IRAS project ID: 212753

Thank you for your letter of 06 October 2016, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Mr Rajat Khullar, nrescommittee.london-cityandeast@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

The Committee noted that the response to question A50 of the IRAS form mentions that the study will not be published. The Committee recommended that if possible the study should be published on a suitable database. You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. This is not a condition of the favourable opinion but a recommendation from the Committee for your consideration.

You should notify the REC once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Revised documents should be submitted to the REC electronically from IRAS. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which you can make available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at <http://www.rdforum.nhs.uk>.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Covering letter on headed paper [cover letter]		10 August 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Clinical Trials Confirmation of cover letter 2016]		10 August 2016
Letter from funder [Funding letter]		22 July 2016
Letter from sponsor [160810 T DONG PID 12224 signed sponsor letter EC]		10 August 2016
Letter from statistician [Support letter from statistician]		16 August 2016
Letters of invitation to participant [Volunteers wanted for for a study to understand the role of immune cells in contributing to cancer development]	V1	10 August 2016
Participant consent form [Consent form]	V1	05 August 2016
Participant information sheet (PIS) [PARTICIPANT INFORMATION SHEET-V2]	V2	06 October 2016
REC Application Form [REC_Form_06102016]		06 October 2016
Referee's report or other scientific critique report [Peer review comment]		12 August 2016
Research protocol or project proposal [Research protocol]	V1	05 August 2016
Summary CV for Chief Investigator (CI)	Tao Dong CV	21 July 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:

<http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance>

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at <http://www.hra.nhs.uk/hra-training/>

16/LO/1607

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely



pp Dr John Keen
Chair

Email: nrescommittee.london-cityandeast@nhs.net

Enclosures: *"After ethical review – guidance for researchers"*

Copy to: *Ms Heather House*

Appendix 4: HBV HCC surgical patients

Patient I.D.	Gender	Age	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc
Patient1	Male	48	+	-	-	+	+
Patient2	Male	51	+	-	-	+	+
Patient3	Male	57	+	-	+	-	+
Patient4	Male	66	+	-	-	-	+
Patient5	Male	55	+	-	-	+	+
Patient6	Male	52	+	-	+	-	+
Patient7	Male	59	+	-	+	-	+
Patient8	Male	56	+	-	-	+	+
Patient9	Male	65	+	-	-	+	+
Patient10	Female	38	+	-	+	-	+
Patient11	Female	52	+	-	-	-	+
Patient12	Male	54	+	-	-	+	+
Patient13	Female	62	+	-	-	+	+
Patient14	Male	42	+	-	+	-	+
Patient15	Male	57	+	-	-	+	+
Patient16	Male	52	+	-	-	+	+
Patient17	Male	41	+	-	-	+	+
Patient18	Female	59	+	-	+	-	+

Patient I.D.	Gender	HBV viral load(IU/ML)	ALT(U/L)	AST(U/L)	TNM classification	BCLC classification
Patient1	Male	7650	65.1	68	II	B
Patient2	Male	859000	41.2	51.4	IIIb	C
Patient3	Male	5560	42	75.8	IIIa	B
Patient4	Male	972	25.7	35.5	IIIa	C
Patient5	Male	below 100	425.4	190.7	IIIb	C
Patient6	Male	625000	329.2	191.9	IIIb	C
Patient7	Male	2560000	54.5	31.6	I	A1
Patient8	Male	below 100	19.4	34.4	II	B
Patient9	Male	31400	9	40	I	A1
Patient10	Female	2300	40	35	IIIb	C
Patient11	Female	4280	43.1	41.9	I	A2
Patient12	Male	below 100	50	15	II	A3
Patient13	Female	431	20.6	23.2	IV	D
Patient14	Male	2350	34.2	60.5	IV	C
Patient15	Male	166	50	40	II	B
Patient16	Male	3450	47	68	II	B
Patient17	Male	3800	37	52	III	C
Patient18	Female	6260	23	39	IIIb	C

Appendix 5: Breast cancer and breast fibroadenoma patients

Stage:	ID:	Gender:	Age:	Diagnosis:
Stage I	244162	Female	68	Breast cancer
Stage I	246078	Female	50	Breast cancer
Stage I	246190	Female	54	Breast cancer
Stage I	246051	Female	69	Breast cancer
Stage I	245630	Female	61	Breast cancer
Stage I	271170	Female	66	Breast cancer
Stage I	272414	Female	50	Breast cancer
Stage I	259062	Female	54	Breast cancer
Stage I	258826	Female	50	Breast cancer
Stage I	199404	Female	46	Breast cancer
Stage II	245837	Female	52	Breast cancer
Stage II	158149	Female	47	Breast cancer
Stage II	246216	Female	36	Breast cancer
Stage II	245975	Female	64	Breast cancer
Stage II	246371	Female	75	Breast cancer
Stage II	246512	Female	40	Breast cancer
Stage II	246705	Female	68	Breast cancer
Stage II	271438	Female	47	Breast cancer
Stage II	271803	Female	51	Breast cancer
Stage II	271735	Female	63	Breast cancer
Stage II	271780	Female	53	Breast cancer
Stage II	258851	Female	74	Breast cancer
Stage II	258746	Female	60	Breast cancer
Stage II	258736	Female	47	Breast cancer
Stage II	258966	Female	44	Breast cancer
Stage II	258970	Female	37	Breast cancer
Stage II	259199	Female	39	Breast cancer
Stage II	259354	Female	41	Breast cancer
Stage II	259350	Female	59	Breast cancer
Stage III	245685	Female	59	Breast cancer
Stage III	246000	Female	45	Breast cancer
Stage III	246072	Female	37	Breast cancer
Stage III	246280	Female	48	Breast cancer
Stage III	246032	Female	46	Breast cancer
Stage III	246443	Female	41	Breast cancer
Stage III	245757	Female	53	Breast cancer
Stage III	246549	Female	49	Breast cancer
Stage III	246722	Female	47	Breast cancer
Stage III	270922	Female	40	Breast cancer
Stage III	271323	Female	57	Breast cancer
Stage III	258745	Female	61	Breast cancer
Stage III	259202	Female	49	Breast cancer

Stage III	259570	Female	40	Breast cancer
-	258656	Female	31	Fibroadenoma
-	258648	Female	37	Fibroadenoma
-	258843	Female	23	Fibroadenoma
-	258856	Female	16	Fibroadenoma
-	259065	Female	21	Fibroadenoma
-	259311	Female	24	Fibroadenoma
-	259411	Female	13	Fibroadenoma
-	259495	Female	13	Fibroadenoma
-	259528	Female	20	Fibroadenoma

Appendix 6: Hepatic haemangioma patients and other cancer patients

ID:	Gender:	Age:	Diagnosis:
270763	Female	75	Colorectal cancer
270683	Female	71	Colorectal cancer
270520	Female	52	Colorectal cancer
271266	Female	67	Colorectal cancer
271155	Male	65	Colorectal cancer
271114	Male	55	Colorectal cancer
271011	Male	78	Colorectal cancer
270870	Male	71	Colorectal cancer
258722	Male	55	Colorectal cancer
258054	Male	46	Colorectal cancer
258677	Male	51	Colorectal cancer
258724	Male	73	Colorectal cancer
257800	Male	76	Colorectal cancer
258541	Male	54	Colorectal cancer
259214	Male	49	Colorectal cancer
259534	Male	22	Colorectal cancer
258159	Female	55	Oesophageal cancer
258290	Female	66	Oesophageal cancer
270567	Male	62	Oesophageal cancer
271053	Male	61	Oesophageal cancer
271699	Male	53	Oesophageal cancer
258789	Male	58	Oesophageal cancer
258783	Male	67	Oesophageal cancer
259032	Male	63	Oesophageal cancer
259473	Male	66	Oesophageal cancer
270962	Female	75	Gastric cancer
257706	Female	50	Gastric cancer
258452	Female	48	Gastric cancer
270747	Male	63	Gastric cancer
270677	Male	63	Gastric cancer
270837	Male	74	Gastric cancer
271730	Male	71	Gastric cancer
272082	Male	45	Gastric cancer
258216	Male	67	Gastric cancer
258110	Male	49	Gastric cancer
258307	Male	67	Gastric cancer
258992	Male	70	Gastric cancer
258000	Male	54	Gastric cancer
270435	Male	55	Gastric cancer
199443	Male	44	HCC
258760	Male	40	HCC

258754	Male	66	HCC
TY389732	Female	55	Hepatic haemangioma
AY32303	Female	64	Hepatic haemangioma
AY39320	Female	48	Hepatic haemangioma
AY39832	Female	52	Hepatic haemangioma
258517	Female	52	Hepatic haemangioma
258773	Female	52	Hepatic haemangioma
259088	Female	44	Hepatic haemangioma
259291	Female	45	Hepatic haemangioma
270869	Female	63	Lung cancer
257957	Female	52	Lung cancer
270197	Male	57	Lung cancer
271362	Male	55	Lung cancer
271359	Male	70	Lung cancer
258762	Male	70	Lung cancer
258640	Male	48	Lung cancer
258128	Male	70	Lung cancer
272338	Female	57	Renal cancer
254131	Female	60	Renal cancer
270562	Male	64	Renal cancer
258273	Male	62	Renal cancer
255520	Male	78	Renal cancer
256783	Male	46	Renal cancer

Appendix 7: Peripheral blood cohort of HBV chronically infected individuals

CHB:					
ID:	Gender:	Age:	ALT(U/L)	AST(U/L)	H B V D N A :IU/ml
'0000020802	Female	28	21.2	20	
1000098321	Female	34	31.5	29.3	7.64E+06
1000224902	Female	28	23.3	19.1	1.58E+06
'0000032705	Female	27	117.4	78.6	3.56E+05
'0000053604	Female	53	17	25.9	<20
282428	Female	36	27.4	20.4	
303047	Male	29	129.5	121.1	7.76E+05
1000276062	Male	54	35.8	26.1	<20
'0000055967	Female	28	15.7	19.9	1.57E+06
1000278546	Female	55	45	24	8.26E+03
382477	Female	29	43	23	1.43E+06
'0000328876	Female	37	14.6	20.4	0.00E+00
'0000276401	Female	37	16.9	0.1	1.81E+03
'0000278929	Female	31	21.5	23.8	<20
'0000249178	Female	34	20.3	19.3	6.77E+01
'0000343548	Male	43	49	43.2	<20
1000170201	Male	46	23.6	28.1	0.00E+00
HBV cirrhosis:					
60230	Male	46	18.7	19	<20
303043	Male	44	27	72.7	9.04E+02
302980	Male	51	49.9	49.2	1.83E+05
302273	Male	48	21.5	40.1	
303697	Male	41	44.9	29.3	
301723	Female	63	14.8	42.3	
303203	Male	59	33.7	37.4	<20
303740	Male	37	12	13.7	
303736	Male	51	117.4	99.9	<100
304085	Female	44	51.3	58.1	2.66E+02
298761	Female	47	18.2	45.7	1.37E+02
HBV HCC:					
302925	Male	48	21.5	15.8	6.54E+02
288979	Male	55	18	16	1.62E+01
303020	Male	68	216.5	876.5	3.58E+03
248189	Male	61	170.8	155.2	2.68E+01
303157	Male	57	27.6	30.6	1.75E+04
302870	Male	56	23.1	31.3	2.62E+01
290268	Male	64	82.8	161.4	7.15E+04

303407	Male	32	723.1	1012.4	4.25E+04
304213	Male	53	167.1	2656.7	5.48E+04
293871	Female	59	20.9	30	3.54E+03
304547	Male	65	55.8	45.6	2.13E+04
303272	Male	26	62.6	40.6	1.57E+05
290593	Male	50	28.9	16.4	<100
303458	Male	52	69.1	68.6	4.15E+04
304134	Male	49	90	79.9	5.08E+04
Healthy donors:					
CMU01	Male	22	None	None	None
CMU02	Male	21	None	None	None
CMU03	Male	23	None	None	None
CMU04	Male	22	None	None	None
CMU05	Male	21	None	None	None
CMU06	Male	22	None	None	None
CMU07	Female	20	None	None	None
CMU08	Female	20	None	None	None
CMU09	Female	22	None	None	None
CMU10	Female	23	None	None	None
CMU11	Female	23	None	None	None
CMU12	Male	21	None	None	None
CMU13	Male	22	None	None	None
CMU14	Male	21	None	None	None
CMU15	Male	23	None	None	None
CMU16	Male	20	None	None	None
CMU17	Male	21	None	None	None
CMU18	Female	22	None	None	None

8 References:

1. El-Serag, H.B., *Epidemiology of viral hepatitis and hepatocellular carcinoma*. Gastroenterology, 2012. **142**(6): p. 1264-1273 e1.
2. El-Serag, H.B. and K.L. Rudolph, *Hepatocellular carcinoma: epidemiology and molecular carcinogenesis*. Gastroenterology, 2007. **132**(7): p. 2557-76.
3. Zhu, A.X., et al., *HCC and angiogenesis: possible targets and future directions*. Nat Rev Clin Oncol, 2011. **8**(5): p. 292-301.
4. El-Serag, H.B., *Current Concepts Hepatocellular Carcinoma* NEJM, 2011.
5. Fattovich, G., *Natural history and prognosis of hepatitis B*. Seminars in Liver Disease 2003. **23**((1)): p. 47-58.
6. Dane, D.S., C.H. Cameron, and M. Briggs, *Virus-like particles in serum of patients with Australia-antigen-associated hepatitis*. Lancet, 1970. **1**(7649): p. 695-8.
7. Robinson, W.S. and L.I. Lutwick, *The virus of hepatitis, type B (first of two parts)*. N Engl J Med, 1976. **295**(21): p. 1168-75.
8. Ganem, D. and A.M. Prince, *Hepatitis B virus infection--natural history and clinical consequences*. N Engl J Med, 2004. **350**(11): p. 1118-29.
9. Klingmuller, U. and H. Schaller, *Hepadnavirus infection requires interaction between the viral pre-S domain and a specific hepatocellular receptor*. Journal of virology, 1993. **67**(12): p. 7414-22.
10. Bruss, V. and D. Ganem, *The role of envelope proteins in hepatitis B virus assembly*. Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(3): p. 1059-63.
11. Milich, D. and T.J. Liang, *Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection*. Hepatology (Baltimore, Md), 2003. **38**(5): p. 1075-86.
12. Valsamakis, A., *Molecular testing in the diagnosis and management of chronic hepatitis B*. Clinical microbiology reviews, 2007. **20**(3): p. 426-39, table of contents.
13. Tsai, W.L. and R.T. Chung, *Viral hepatocarcinogenesis*. Oncogene, 2010. **29**(16): p. 2309-24.
14. Lupberger, J. and E. Hildt, *Hepatitis B virus-induced oncogenesis*. World journal of gastroenterology, 2007. **13**(1): p. 74-81.
15. Pan, C.Q. and J.X. Zhang, *Natural History and Clinical Consequences of Hepatitis B Virus Infection*. Int J Med Sci, 2005. **2**(1): p. 36-40.
16. Chu, C.-J., et al., *Prevalence of HBV precore/core promoter variants in the United States*. Hepatology (Baltimore, Md), 2003. **38**(3): p. 619-28.
17. Chu, C.-J. and A.S.F. Lok, *Clinical significance of hepatitis B virus genotypes*. Hepatology (Baltimore, Md), 2002. **35**(5): p. 1274-6.
18. Kao, J.-H., et al., *Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection*. Journal of clinical microbiology, 2002. **40**(4): p. 1207-9.
19. Kidd-Ljunggren, K., Y. Miyakawa, and A.H. Kidd, *Genetic variability in hepatitis B viruses*. The Journal of general virology, 2002. **83**(Pt 6): p. 1267-80.

20. Sumi, H., et al., *Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease*. Hepatology (Baltimore, Md), 2003. **37**(1): p. 19-26.
21. Yuan, J., et al., *Hepatitis B virus (HBV) genotypes/subgenotypes in China: mutations in core promoter and precore/core and their clinical implications*. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2007. **39**(2): p. 87-93.
22. Wang, Z., et al., *Hepatitis B virus genotypes and subgenotypes in China*. Hepatology research : the official journal of the Japan Society of Hepatology, 2007. **37**(s1): p. S36-41.
23. Wang, Z., et al., *Distribution and characteristics of hepatitis B virus genotype C subgenotypes in China*. Journal of viral hepatitis, 2007. **14**(6): p. 426-34.
24. Zhong, Y.-W., et al., *Virologic and clinical characteristics of HBV genotypes/subgenotypes in 487 Chinese pediatric patients with CHB*. BMC infectious diseases, 2011. **11**: p. 262.
25. Li, X.-D., et al., *Characterization of hepatitis B virus genotypes/subgenotypes in 1,301 patients with chronic hepatitis B in North China*. Chinese medical journal, 2011. **124**(24): p. 4178-83.
26. Hoofnagle, J.H., *Serologic markers of hepatitis B virus infection*. Annual review of medicine, 1981. **32**: p. 1-11.
27. Ribeiro, R.M., A. Lo, and A.S. Perelson, *Dynamics of hepatitis B virus infection*. Microbes and infection, 2002. **4**(8): p. 829-35.
28. Dusheiko, G. and N. Antonakopoulos, *Current treatment of hepatitis B*. Gut, 2008. **57**(1): p. 105-24.
29. Bengsch, B., B. Martin, and R. Thimme, *Restoration of HBV-specific CD8+ T cell function by PD-1 blockade in inactive carrier patients is linked to T cell differentiation*. J Hepatol, 2014. **61**(6): p. 1212-9.
30. Gallot, D., et al., *[Serum alpha-fetoprotein (AFP) after primary liver cancer resection in adult value of its determination in the detection of recurrence]*. Nouv Presse Med, 1974. **3**(36): p. 2305-8.
31. Qiao, S.S., et al., *Simultaneous measurements of serum AFP, GPC-3 and HCCR for diagnosing hepatocellular carcinoma*. Hepatogastroenterology, 2011. **58**(110-111): p. 1718-24.
32. Wu, Y., H. Liu, and H. Ding, *GPC-3 in hepatocellular carcinoma: current perspectives*. J Hepatocell Carcinoma, 2016. **3**: p. 63-67.
33. Zou, Z.Q., et al., *Gpc-3 is a notable diagnostic, prognostic and a latent targeted therapy marker in hepatocellular carcinoma*. Hepatogastroenterology, 2010. **57**(102-103): p. 1285-90.
34. Esfandiary, A. and S. Ghafouri-Fard, *New York esophageal squamous cell carcinoma-1 and cancer immunotherapy*. Immunotherapy, 2015. **7**(4): p. 411-39.
35. Ayyoub, M., et al., *Tumor-reactive, SSX-2-specific CD8+ T cells are selectively expanded during immune responses to antigen-expressing tumors in melanoma patients*. Cancer Res, 2003. **63**(17): p. 5601-6.
36. Bricard, G., et al., *Naturally acquired Mage-A10- and SSX-2-specific CD8+ T cell responses in patients with hepatocellular carcinoma*. J Immunol, 2005. **174**(3): p. 1709-16.

37. Napoletano, C., et al., *MAGE-A and NY-ESO-1 expression in cervical cancer: prognostic factors and effects of chemotherapy*. Am J Obstet Gynecol, 2008. **198**(1): p. 99 e1-7.
38. Haier, J., et al., *Expression of MAGE-A cancer/testis antigens in esophageal squamous cell carcinomas*. Anticancer Res, 2006. **26**(3B): p. 2281-7.
39. Hamai, A., et al., *Expression of MAGE-A antigens is frequent in triple-negative breast cancers but does not correlate with that of basal-like markers*. Ann Oncol, 2011. **22**(4): p. 986-7.
40. Luetkens, T., et al., *Functional autoantibodies against SSX-2 and NY-ESO-1 in multiple myeloma patients after allogeneic stem cell transplantation*. Cancer Immunol Immunother, 2014. **63**(11): p. 1151-62.
41. Matkovic, B., et al., *Expression of MAGE-A and NY-ESO-1 cancer/testis antigens in medullary breast cancer: retrospective immunohistochemical study*. Croat Med J, 2011. **52**(2): p. 171-7.
42. Montler, R., et al., *OX40, PD-1 and CTLA-4 are selectively expressed on tumor-infiltrating T cells in head and neck cancer*. Clin Transl Immunology, 2016. **5**(4): p. e70.
43. Tumeh, P.C., et al., *PD-1 blockade induces responses by inhibiting adaptive immune resistance*. Nature, 2014. **515**(7528): p. 568-71.
44. Hara, T., H. Miyake, and M. Fujisawa, *Expression pattern of immune checkpoint-associated molecules in radical nephrectomy specimens as a prognosticator in patients with metastatic renal cell carcinoma treated with tyrosine kinase inhibitors*. Urol Oncol, 2017.
45. Kluger, H.M., et al., *PD-L1 Studies Across Tumor Types, its Differential Expression and Predictive Value in Patients Treated with Immune Checkpoint Inhibitors*. Clin Cancer Res, 2017.
46. Roh, J., et al., *Expression of immune checkpoint molecule vsig4 is correlated with poor prognosis in multiple myeloma*. Pathology, 2016. **48 Suppl 1**: p. S153-S154.
47. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma*. N Engl J Med, 2010. **363**(8): p. 711-23.
48. Chen, L. and X. Han, *Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future*. The Journal of clinical investigation, 2015. **125**(9): p. 3384-91.
49. Robert, C., et al., *Nivolumab in previously untreated melanoma without BRAF mutation*. N Engl J Med, 2015. **372**(4): p. 320-30.
50. Topalian, S.L., et al., *Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab*. J Clin Oncol, 2014. **32**(10): p. 1020-30.
51. Chow, L.Q., et al., *Antitumor Activity of Pembrolizumab in Biomarker-Unselected Patients With Recurrent and/or Metastatic Head and Neck Squamous Cell Carcinoma: Results From the Phase Ib KEYNOTE-012 Expansion Cohort*. J Clin Oncol, 2016.
52. Garon, E.B., et al., *Pembrolizumab for the treatment of non-small-cell lung cancer*. N Engl J Med, 2015. **372**(21): p. 2018-28.
53. Muro, K., et al., *Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial*. Lancet Oncol, 2016. **17**(6): p. 717-26.

54. Ansell, S.M., et al., *PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma*. N Engl J Med, 2015. **372**(4): p. 311-9.
55. Nanda, R., et al., *Pembrolizumab in Patients With Advanced Triple-Negative Breast Cancer: Phase Ib KEYNOTE-012 Study*. J Clin Oncol, 2016. **34**(21): p. 2460-7.
56. Okazaki, T. and T. Honjo, *PD-1 and PD-1 ligands: from discovery to clinical application*. Int Immunol, 2007. **19**(7): p. 813-24.
57. Ishida, Y., et al., *Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death*. EMBO J, 1992. **11**(11): p. 3887-95.
58. Dong, H., et al., *B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion*. Nat Med, 1999. **5**(12): p. 1365-9.
59. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation*. J Exp Med, 2000. **192**(7): p. 1027-34.
60. Dong, H., et al., *B7-H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes*. Immunity, 2004. **20**(3): p. 327-36.
61. Latchman, Y., et al., *PD-L2 is a second ligand for PD-1 and inhibits T cell activation*. Nat Immunol, 2001. **2**(3): p. 261-8.
62. Tseng, S.Y., et al., *B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells*. J Exp Med, 2001. **193**(7): p. 839-46.
63. Park, J.J., et al., *B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance*. Blood, 2010. **116**(8): p. 1291-8.
64. Butte, M.J., et al., *Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses*. Immunity, 2007. **27**(1): p. 111-22.
65. Xiao, Y., et al., *RGMb is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance*. J Exp Med, 2014. **211**(5): p. 943-59.
66. Agata, Y., et al., *Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes*. Int Immunol, 1996. **8**(5): p. 765-72.
67. Nishimura, H., et al., *Developmentally regulated expression of the PD-1 protein on the surface of double-negative (CD4-CD8-) thymocytes*. Int Immunol, 1996. **8**(5): p. 773-80.
68. Kroy, D.C., et al., *Liver environment and HCV replication affect human T-cell phenotype and expression of inhibitory receptors*. Gastroenterology, 2014. **146**(2): p. 550-61.
69. Ohaegbulam, K.C., et al., *Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway*. Trends Mol Med, 2015. **21**(1): p. 24-33.
70. Ribas, A., *Tumor immunotherapy directed at PD-1*. N Engl J Med, 2012. **366**(26): p. 2517-9.
71. Taube, J.M., et al., *Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape*. Sci Transl Med, 2012. **4**(127): p. 127ra37.
72. Velcheti, V., et al., *Programmed death ligand-1 expression in non-small cell lung cancer*. Lab Invest, 2014. **94**(1): p. 107-16.

73. Shu, C.A. and N.A. Rizvi, *Into the Clinic With Nivolumab and Pembrolizumab*. Oncologist, 2016. **21**(5): p. 527-8.
74. Brahmer, J.R., et al., *Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates*. J Clin Oncol, 2010. **28**(19): p. 3167-75.
75. Topalian, S.L., et al., *Safety, activity, and immune correlates of anti-PD-1 antibody in cancer*. The New England journal of medicine, 2012. **366**(26): p. 2443-54.
76. Sharma, P., et al., *Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate 275): a multicentre, single-arm, phase 2 trial*. Lancet Oncol, 2017.
77. Hamid, O., et al., *Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma*. N Engl J Med, 2013. **369**(2): p. 134-44.
78. Robert, C., et al., *Pembrolizumab versus Ipilimumab in Advanced Melanoma*. N Engl J Med, 2015. **372**(26): p. 2521-32.
79. *Pembrolizumab Has Antitumor Activity In Urothelial Cancer*. Cancer Discov, 2017. **7**(3): p. OF5.
80. Bellmunt, J., et al., *Pembrolizumab as Second-Line Therapy for Advanced Urothelial Carcinoma*. N Engl J Med, 2017.
81. Sorscher, S., *Pembrolizumab in Non-Small-Cell Lung Cancer*. N Engl J Med, 2017. **376**(10): p. 996-7.
82. Plimack, E.R., et al., *Safety and activity of pembrolizumab in patients with locally advanced or metastatic urothelial cancer (KEYNOTE-012): a non-randomised, open-label, phase 1b study*. Lancet Oncol, 2017. **18**(2): p. 212-220.
83. Cha, E., J. Wallin, and M. Kowanetz, *PD-L1 inhibition with MPDL3280A for solid tumors*. Semin Oncol, 2015. **42**(3): p. 484-7.
84. Powles, T., et al., *MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer*. Nature, 2014. **515**(7528): p. 558-62.
85. Herbst, R.S., et al., *Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients*. Nature, 2014. **515**(7528): p. 563-7.
86. Brunet, J.F., et al., *A new member of the immunoglobulin superfamily--CTLA-4*. Nature, 1987. **328**(6127): p. 267-70.
87. Brunet, J.F., *A new member of the immunoglobulin superfamily-CTLA-4*. European journal of Immunology, 1987.
88. Linsley, *CTLA-4 is a second receptor for the B cell activation antigen B7*. Journal of experimental medicine, 1991.
89. Linsley, *Coexpression and Functional Cooperation of CTLA-4 and CD28 on Activated T Lymphocytes*. Journal of experimental medicine, 1992.
90. Bashyam, H., *CTLA-4: From conflict to clinic*. J Exp Med, 2007. **204**(6): p. 1243.
91. Allison, M.K.a.J., *CD28 and CTLA-4 Have Opposing Effects on the Response of T cells to Stimulation*. Journal of experimental medicine, 1995.
92. Elizabeth A. Tivol, F.B., ' and W.P.L. A. Nicola Schweitzer*, ' Jeffrey A. Bluestone,t and Arlene H. Sharpe, *Loss of CTLA-4 Leads to Massive Lymphoproliferation and Fatal Multiorgan Tissue Destruction, Revealing a Critical Negative Regulatory Role of CTLA-4*. 1995.

93. Azuma, M., et al., *B70 antigen is a second ligand for CTLA-4 and CD28*. Nature, 1993. **366**(6450): p. 76-9.
94. Freeman, G.J., et al., *Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice*. Science, 1993. **262**(5135): p. 907-9.
95. Hathcock, K.S., et al., *Identification of an alternative CTLA-4 ligand costimulatory for T cell activation*. Science, 1993. **262**(5135): p. 905-7.
96. Leach, D.R., M.F. Krummel, and J.P. Allison, *Enhancement of antitumor immunity by CTLA-4 blockade*. Science, 1996. **271**(5256): p. 1734-6.
97. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy*. Nat Rev Cancer, 2012. **12**(4): p. 252-64.
98. O'Day, S.J., O. Hamid, and W.J. Urba, *Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4): a novel strategy for the treatment of melanoma and other malignancies*. Cancer, 2007. **110**(12): p. 2614-27.
99. Sarnaik, A.A. and J.S. Weber, *Recent advances using anti-CTLA-4 for the treatment of melanoma*. Cancer J, 2009. **15**(3): p. 169-73.
100. Weber, J., *Overcoming immunologic tolerance to melanoma: targeting CTLA-4 with ipilimumab (MDX-010)*. Oncologist, 2008. **13 Suppl 4**: p. 16-25.
101. Yuan, J., et al., *CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit*. Proc Natl Acad Sci U S A, 2008. **105**(51): p. 20410-5.
102. Lipson, E.J. and C.G. Drake, *Ipilimumab: an anti-CTLA-4 antibody for metastatic melanoma*. Clin Cancer Res, 2011. **17**(22): p. 6958-62.
103. Tarhini, A.A. and J.M. Kirkwood, *CTLA-4-blocking immunotherapy with ipilimumab for advanced melanoma*. Oncology (Williston Park), 2010. **24**(14): p. 1302, 1304.
104. Camacho, L.H., *CTLA-4 blockade with ipilimumab: biology, safety, efficacy, and future considerations*. Cancer Med, 2015. **4**(5): p. 661-72.
105. Downey, S.G., et al., *Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade*. Clin Cancer Res, 2007. **13**(22 Pt 1): p. 6681-8.
106. Monney, L., et al., *Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease*. Nature, 2002. **415**(6871): p. 536-41.
107. Anderson, A.C., N. Joller, and V.K. Kuchroo, *Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation*. Immunity, 2016. **44**(5): p. 989-1004.
108. Zhu, C., et al., *The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity*. Nat Immunol, 2005. **6**(12): p. 1245-52.
109. DeKruyff, G.J.F.J.M.C.D.T.U.R.H., *TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity*. Immunological review, 2010.
110. DeKruyff, R.H., et al., *T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells*. J Immunol, 2010. **184**(4): p. 1918-30.
111. Huang, Y.H., et al., *CEACAM1 regulates TIM-3-mediated tolerance and exhaustion*. Nature, 2015. **517**(7534): p. 386-90.
112. Huang, Y.H., et al., *Corrigendum: CEACAM1 regulates TIM-3-mediated tolerance and exhaustion*. Nature, 2016. **536**(7616): p. 359.

113. Tang, D. and M.T. Lotze, *Tumor immunity times out: TIM-3 and HMGB1*. Nat Immunol, 2012. **13**(9): p. 808-10.
114. Jones, R.B., et al., *Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection*. J Exp Med, 2008. **205**(12): p. 2763-79.
115. Li, H., et al., *Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma*. Hepatology, 2012. **56**(4): p. 1342-51.
116. McMahan, R.H., et al., *Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity*. J Clin Invest, 2010. **120**(12): p. 4546-57.
117. Vali, B., et al., *HCV-specific T cells in HCV/HIV co-infection show elevated frequencies of dual Tim-3/PD-1 expression that correlate with liver disease progression*. Eur J Immunol, 2010. **40**(9): p. 2493-505.
118. Wu, W., et al., *Tim-3 expression on peripheral T cell subsets correlates with disease progression in hepatitis B infection*. Virol J, 2011. **8**: p. 113.
119. Jin, H.T., et al., *Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection*. Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14733-8.
120. Ju, Y., et al., *Blockade of Tim-3 pathway ameliorates interferon-gamma production from hepatic CD8+ T cells in a mouse model of hepatitis B virus infection*. Cell Mol Immunol, 2009. **6**(1): p. 35-43.
121. Sakuishi, K., et al., *Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity*. J Exp Med, 2010. **207**(10): p. 2187-94.
122. Zaretsky, J.M., et al., *Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma*. N Engl J Med, 2016. **375**(9): p. 819-29.
123. Koyama, S., et al., *Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints*. Nat Commun, 2016. **7**: p. 10501.
124. Blaser, C., M. Kaufmann, and H. Pircher, *Virus-activated CD8 T cells and lymphokine-activated NK cells express the mast cell function-associated antigen, an inhibitory C-type lectin*. J Immunol, 1998. **161**(12): p. 6451-4.
125. Voehringer, D., M. Koschella, and H. Pircher, *Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1)*. Blood, 2002. **100**(10): p. 3698-702.
126. Fletcher, J.M., et al., *Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion*. J Immunol, 2005. **175**(12): p. 8218-25.
127. Gothert, J.R., et al., *Expanded CD8+ T cells of murine and human CLL are driven into a senescent KLRG1+ effector memory phenotype*. Cancer Immunol Immunother, 2013. **62**(11): p. 1697-1709.
128. Grundemann, C., et al., *The NK receptor KLRG1 is dispensable for virus-induced NK and CD8+ T-cell differentiation and function in vivo*. Eur J Immunol, 2010. **40**(5): p. 1303-14.
129. Ye, F., J. Turner, and E. Flano, *Contribution of pulmonary KLRG1(high) and KLRG1(low) CD8 T cells to effector and memory responses during influenza virus infection*. J Immunol, 2012. **189**(11): p. 5206-11.

130. Thimme, R., et al., *Increased expression of the NK cell receptor KLRG1 by virus-specific CD8 T cells during persistent antigen stimulation.* J Virol, 2005. **79**(18): p. 12112-6.
131. Hofmann, M., O. Schweier, and H. Pircher, *Different inhibitory capacities of human and mouse KLRG1 are linked to distinct disulfide-mediated oligomerizations.* Eur J Immunol, 2012. **42**(9): p. 2484-90.
132. Ito, M., et al., *Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity.* J Exp Med, 2006. **203**(2): p. 289-95.
133. Carsten Gründemann, M.B., Oliver Schweier, Nanette von Oppen, Ute Lässig, Philippe Saudan, Karl-Friedrich Becker, Klaus Karp, Thomas Hanke, Martin F. Bachmann and Hanspeter Pircher, *Cutting Edge: Identification of E-Cadherin as a Ligand for the Murine Killer Cell Lectin-Like Receptor G1.* Journal of immunology 2006.
134. Marcolino, I., et al., *Frequent expression of the natural killer cell receptor KLRG1 in human cord blood T cells: correlation with replicative history.* Eur J Immunol, 2004. **34**(10): p. 2672-80.
135. Henson, S.M. and A.N. Akbar, *KLRG1--more than a marker for T cell senescence.* Age (Dordr), 2009. **31**(4): p. 285-91.
136. Malaise, M., et al., *KLRG1+ NK cells protect T-bet-deficient mice from pulmonary metastatic colorectal carcinoma.* J Immunol, 2014. **192**(4): p. 1954-61.
137. de Jong, R., et al., *Regulation of T-cell differentiation by CD2 and CD28 accessory molecules.* Immunology, 1991. **74**(2): p. 175-82.
138. Hombach, A., et al., *Specific activation of resting T cells against tumour cells by bispecific antibodies and CD28-mediated costimulation is accompanied by Th1 differentiation and recruitment of MHC-independent cytotoxicity.* Clin Exp Immunol, 1997. **108**(2): p. 352-7.
139. Tessmer, M.S., et al., *KLRG1 binds cadherins and preferentially associates with SHIP-1.* Int Immunol, 2007. **19**(4): p. 391-400.
140. Henson, S.M., et al., *KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells.* Blood, 2009. **113**(26): p. 6619-28.
141. Bengsch, B., et al., *Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation.* PLoS Pathog, 2010. **6**(6): p. e1000947.
142. Baitsch, L., et al., *Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients.* J Clin Invest, 2011. **121**(6): p. 2350-60.
143. Li, L., et al., *KLRG1 restricts memory T cell antitumor immunity.* Oncotarget, 2016. **7**(38): p. 61670-61678.
144. Mathew, P.A., et al., *Cloning and characterization of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated natural killer cells and T cells.* J Immunol, 1993. **151**(10): p. 5328-37.
145. Chlewicki, L.K., et al., *Molecular basis of the dual functions of 2B4 (CD244).* J Immunol, 2008. **180**(12): p. 8159-67.
146. Garni-Wagner, B.A., et al., *A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells.* J Immunol, 1993. **151**(1): p. 60-70.

147. Lee, K.M., et al., *2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells*. J Exp Med, 2004. **199**(9): p. 1245-54.
148. Vaidya, S.V., et al., *Targeted disruption of the 2B4 gene in mice reveals an in vivo role of 2B4 (CD244) in the rejection of B16 melanoma cells*. J Immunol, 2005. **174**(2): p. 800-7.
149. Messmer, B., et al., *CD48 stimulation by 2B4 (CD244)-expressing targets activates human NK cells*. J Immunol, 2006. **176**(8): p. 4646-50.
150. Aldy, K.N., et al., *2B4+ CD8+ T cells play an inhibitory role against constrained HIV epitopes*. Biochem Biophys Res Commun, 2011. **405**(3): p. 503-7.
151. Pombo, C., et al., *Elevated Expression of CD160 and 2B4 Defines a Cytolytic HIV-Specific CD8+ T-Cell Population in Elite Controllers*. J Infect Dis, 2015. **212**(9): p. 1376-86.
152. Stanitsky, N., et al., *The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity*. Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17858-63.
153. Yu, X., et al., *The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells*. Nat Immunol, 2009. **10**(1): p. 48-57.
154. Joller, N., et al., *Cutting edge: TIGIT has T cell-intrinsic inhibitory functions*. J Immunol, 2011. **186**(3): p. 1338-42.
155. Casado, J.G., et al., *Expression of adhesion molecules and ligands for activating and costimulatory receptors involved in cell-mediated cytotoxicity in a large panel of human melanoma cell lines*. Cancer Immunol Immunother, 2009. **58**(9): p. 1517-26.
156. Cristina Bottino, R.C., Daniela Pende, Paola Rivera, and B.C. Marina Nanni, Claudia Cantoni, Jessica Grassi, Stefania Marcenaro, Nicolas Reymond, Massimo Vitale, Lorenzo Moretta, Marc Lopez, and Alessandro Moretta, *Identification of PVR (CD155) and Nectin-2 (CD112) as Cell Surface Ligands for the Human DNAM-1 (CD226) Activating Molecule*. Journal of experimental medicine, 2003.
157. Chan, C.J., et al., *The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions*. Nat Immunol, 2014. **15**(5): p. 431-8.
158. Iguchi-Manaka, A., et al., *Accelerated tumor growth in mice deficient in DNAM-1 receptor*. J Exp Med, 2008. **205**(13): p. 2959-64.
159. Gilfillan, S., et al., *DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors*. J Exp Med, 2008. **205**(13): p. 2965-73.
160. Kurtulus, S., et al., *TIGIT predominantly regulates the immune response via regulatory T cells*. J Clin Invest, 2015. **125**(11): p. 4053-62.
161. Chauvin, J.M., et al., *TIGIT and PD-1 impair tumor antigen-specific CD8(+) T cells in melanoma patients*. J Clin Invest, 2015. **125**(5): p. 2046-58.
162. A. BENSUSSAN, E.G., S. EL MARSAFY, V. SCHIAVON, I.-G. MANSUR, J. DAUSSET, L. BOUMSELL, AND E. CAROSELLA, *BY55 monoclonal antibody delineates within human cord blood and bone marrow lymphocytes distinct cell subsets mediating cytotoxic activity*. Proc. Nati.Acad. Sci., 1994.

163. Nikolova, M.H., et al., *The CD160+ CD8high cytotoxic T cell subset correlates with response to HAART in HIV-1+ patients*. Cell Immunol, 2005. **237**(2): p. 96-105.
164. Tsujimura, K., et al., *Characterization of murine CD160+ CD8+ T lymphocytes*. Immunol Lett, 2006. **106**(1): p. 48-56.
165. Fons, P., et al., *Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and by direct binding to CD160 receptor expressed by endothelial cells*. Blood, 2006. **108**(8): p. 2608-15.
166. Barakonyi, A., et al., *Cutting edge: engagement of CD160 by its HLA-C physiological ligand triggers a unique cytokine profile secretion in the cytotoxic peripheral blood NK cell subset*. J Immunol, 2004. **173**(9): p. 5349-54.
167. Giustiniani, J., A. Marie-Cardine, and A. Bensussan, *A soluble form of the MHC class I-specific CD160 receptor is released from human activated NK lymphocytes and inhibits cell-mediated cytotoxicity*. J Immunol, 2007. **178**(3): p. 1293-300.
168. Riley, J.L. and C.H. June, *The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation*. Blood, 2005. **105**(1): p. 13-21.
169. Otsuki, N., et al., *Expression and function of the B and T lymphocyte attenuator (BTLA/CD272) on human T cells*. Biochem Biophys Res Commun, 2006. **344**(4): p. 1121-7.
170. Kaye, J., *CD160 and BTLA: LIGHTs out for CD4+ T cells*. Nat Immunol, 2008. **9**(2): p. 122-4.
171. Mahoney, K.M., P.D. Rennert, and G.J. Freeman, *Combination cancer immunotherapy and new immunomodulatory targets*. Nat Rev Drug Discov, 2015. **14**(8): p. 561-84.
172. Murphy, K.M., C.A. Nelson, and J.R. Sedy, *Balancing co-stimulation and inhibition with BTLA and HVEM*. Nat Rev Immunol, 2006. **6**(9): p. 671-81.
173. Gavrieli, M., et al., *BTLA and HVEM cross talk regulates inhibition and costimulation*. Adv Immunol, 2006. **92**: p. 157-85.
174. Haymaker, C., et al., *PD-1 and BTLA and CD8(+) T-cell "exhaustion" in cancer: "Exercising" an alternative viewpoint*. Oncoimmunology, 2012. **1**(5): p. 735-738.
175. Freeman, G.C.G.J., *The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation*. Immunological reviews, 2009.
176. Watanabe, N., et al., *BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1*. Nat Immunol, 2003. **4**(7): p. 670-9.
177. Paulos, C.M. and C.H. June, *Putting the brakes on BTLA in T cell-mediated cancer immunotherapy*. J Clin Invest, 2010. **120**(1): p. 76-80.
178. Derre, L., et al., *BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination*. J Clin Invest, 2010. **120**(1): p. 157-67.
179. Nirschl, C.J. and C.G. Drake, *Molecular pathways: coexpression of immune checkpoint molecules: signaling pathways and implications for cancer immunotherapy*. Clin Cancer Res, 2013. **19**(18): p. 4917-24.
180. Chuang, E., et al., *The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A*. Immunity, 2000. **13**(3): p. 313-22.

181. Marengere, L.E., et al., *Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4*. Science, 1996. **272**(5265): p. 1170-3.
182. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms*. Mol Cell Biol, 2005. **25**(21): p. 9543-53.
183. Chemnitz, J.M., et al., *SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation*. J Immunol, 2004. **173**(2): p. 945-54.
184. Sheppard, K.A., et al., *PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta*. FEBS Lett, 2004. **574**(1-3): p. 37-41.
185. van de Weyer, P.S., et al., *A highly conserved tyrosine of Tim-3 is phosphorylated upon stimulation by its ligand galectin-9*. Biochem Biophys Res Commun, 2006. **351**(2): p. 571-6.
186. Rangachari, M., et al., *Bat3 promotes T cell responses and autoimmunity by repressing Tim-3-mediated cell death and exhaustion*. Nat Med, 2012. **18**(9): p. 1394-400.
187. Kurachi, J.W.a.M., *molecular and cellular insights into T cell exhaustion*. nature reviews immunology, 2015.
188. Curry, M.P., *Isolation of lymphocytes from normal adult human liver suitable for phenotypic and functional characterisation*. journal of Immunological Method, 2000.
189. Blom, K.G., et al., *Isolation of murine intrahepatic immune cells employing a modified procedure for mechanical disruption and functional characterization of the B, T and natural killer T cells obtained*. Clin Exp Immunol, 2009. **155**(2): p. 320-9.
190. Davis, A.S., et al., *Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Human Respiratory Tissue*. J Histochem Cytochem, 2014. **62**(6): p. 405-423.
191. Viegas, M.S., et al., *An improved and cost-effective methodology for the reduction of autofluorescence in direct immunofluorescence studies on formalin-fixed paraffin-embedded tissues*. Eur J Histochem, 2007. **51**(1): p. 59-66.
192. Amir el, A.D., et al., *viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia*. Nat Biotechnol, 2013. **31**(6): p. 545-52.
193. Hotchkiss, R.S. and L.L. Moldawer, *Parallels between cancer and infectious disease*. N Engl J Med, 2014. **371**(4): p. 380-3.
194. Pauken, K.E. and E.J. Wherry, *Overcoming T cell exhaustion in infection and cancer*. Trends Immunol, 2015. **36**(4): p. 265-76.
195. Grosso, J.F. and M.N. Jure-Kunkel, *CTLA-4 blockade in tumor models: an overview of preclinical and translational research*. Cancer Immun, 2013. **13**: p. 5.
196. Garon, E.B., et al., *Pembrolizumab for the treatment of non-small-cell lung cancer*. The New England journal of medicine, 2015. **372**(21): p. 2018-28.
197. Nghiem, P.T., et al., *PD-1 Blockade with Pembrolizumab in Advanced Merkel-Cell Carcinoma*. N Engl J Med, 2016. **374**(26): p. 2542-52.

198. Sullivan, R.J. and K.T. Flaherty, *Pembrolizumab for Treatment of Patients with Advanced or Unresectable Melanoma*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2015. **21**(13): p. 2892-7.
199. Kahan, S.M., E.J. Wherry, and A.J. Zajac, *T cell exhaustion during persistent viral infections*. Virology, 2015. **479-480**: p. 180-93.
200. Wherry, E.J., *T cell exhaustion*. Nature Immunology, 2011. **13**(6): p. 492-499.
201. Baitsch, L., et al., *Extended co-expression of inhibitory receptors by human CD8 T-cells depending on differentiation, antigen-specificity and anatomical localization*. PLoS One, 2012. **7**(2): p. e30852.
202. Ye, B., et al., *T-cell exhaustion in chronic hepatitis B infection: current knowledge and clinical significance*. Cell Death Dis, 2015. **6**: p. e1694.
203. Matsushita, H., et al., *Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting*. Nature, 2012. **482**(7385): p. 400-4.
204. Shuai, Z., et al., *Adaptive immunity in the liver*. Cell Mol Immunol, 2016. **13**(3): p. 354-68.
205. *Pembrolizumab shows potential in breast cancer*. Cancer Discov, 2015. **5**(2): p. 100-1.
206. Silvia A. Fuertes Marraco, N.J.N., Grégory Verdeil and Daniel E. Speiser, *inhibitory receptors beyond T cell exhaustion*. Frontiers in Immunology, 2015.
207. Hu, M. and K. Polyak, *Microenvironmental regulation of cancer development*. Curr Opin Genet Dev, 2008. **18**(1): p. 27-34.
208. Zhang, Y. and L. Chen, *Classification of Advanced Human Cancers Based on Tumor Immunity in the MicroEnvironment (TIME) for Cancer Immunotherapy*. JAMA Oncol, 2016. **2**(11): p. 1403-1404.
209. Jiang, P., et al., *Effective targeting of the tumor microenvironment for cancer therapy*. Anticancer Res, 2012. **32**(4): p. 1203-12.
210. Bhaijee, F. and R.A. Anders, *PD-L1 Expression as a Predictive Biomarker: Is Absence of Proof the Same as Proof of Absence?* JAMA Oncol, 2016. **2**(1): p. 54-5.
211. Rachel H. McMahan, L.G.-M., Michael I. Nishimura, Brian J. McMahon, Michael Kemper, Todd M. Allen, David R. Gretch, and Hugo R. Rosen, *Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity*. Journal of clinical investigation, 2010.
212. Prieto, P.A., et al., *CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma*. Clin Cancer Res, 2012. **18**(7): p. 2039-47.
213. Angulo, J.C., et al., *The value of tumour spread, grading and growth pattern as morphological predictive parameters in bladder carcinoma. A critical revision of the 1987 TNM classification*. J Cancer Res Clin Oncol, 1993. **119**(10): p. 578-93.
214. Legat, A., et al., *Inhibitory Receptor Expression Depends More Dominantly on Differentiation and Activation than "Exhaustion" of Human CD8 T Cells*. Front Immunol, 2013. **4**: p. 455.
215. Corrigan-Curay, J., et al., *T-cell immunotherapy: looking forward*. Mol Ther, 2014. **22**(9): p. 1564-74.

216. Kohrt, H.E., et al., *Immunodynamics: a cancer immunotherapy trials network review of immune monitoring in immuno-oncology clinical trials*. J Immunother Cancer, 2016. **4**: p. 15.
217. Larkin, J., et al., *Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma*. N Engl J Med, 2015. **373**(1): p. 23-34.
218. Larkin, J., F.S. Hodi, and J.D. Wolchok, *Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma*. N Engl J Med, 2015. **373**(13): p. 1270-1.
219. Valsecchi, M.E., *Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma*. N Engl J Med, 2015. **373**(13): p. 1270.
220. Roederer, M., J.L. Nozzi, and M.C. Nason, *SPICE: exploration and analysis of post-cytometric complex multivariate datasets*. Cytometry A, 2011. **79**(2): p. 167-74.
221. Rahir, G. and M. Moser, *Tumor microenvironment and lymphocyte infiltration*. Cancer Immunol Immunother, 2012. **61**(6): p. 751-9.
222. Takemoto, N., et al., *The correlation of microsatellite instability and tumor-infiltrating lymphocytes in hereditary non-polyposis colorectal cancer (HNPCC) and sporadic colorectal cancers: the significance of different types of lymphocyte infiltration*. Jpn J Clin Oncol, 2004. **34**(2): p. 90-8.
223. Wherry, E.J. and M. Kurachi, *Molecular and cellular insights into T cell exhaustion*. Nat Rev Immunol, 2015. **15**(8): p. 486-99.
224. Masopust, D. and J.M. Schenkel, *The integration of T cell migration, differentiation and function*. Nat Rev Immunol, 2013. **13**(5): p. 309-20.
225. Virgin, H.W., E.J. Wherry, and R. Ahmed, *Redefining chronic viral infection*. Cell, 2009. **138**(1): p. 30-50.
226. Schenkel, J.M. and D. Masopust, *Tissue-resident memory T cells*. Immunity, 2014. **41**(6): p. 886-97.
227. Geginat, J., A. Lanzavecchia, and F. Sallusto, *Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines*. Blood, 2003. **101**(11): p. 4260-6.
228. Appay, V., et al., *Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections*. Nat Med, 2002. **8**(4): p. 379-85.
229. Kaech, S.M. and W. Cui, *Transcriptional control of effector and memory CD8+ T cell differentiation*. Nat Rev Immunol, 2012. **12**(11): p. 749-61.
230. Pollizzi, K.N. and J.D. Powell, *Integrating canonical and metabolic signalling programmes in the regulation of T cell responses*. Nat Rev Immunol, 2014. **14**(7): p. 435-46.
231. Appay, V., et al., *Phenotype and function of human T lymphocyte subsets: consensus and issues*. Cytometry A, 2008. **73**(11): p. 975-83.
232. Beier, K.C., T. Kallinich, and E. Hamelmann, *Master switches of T-cell activation and differentiation*. Eur Respir J, 2007. **29**(4): p. 804-12.
233. Hato, T., et al., *Immune checkpoint blockade in hepatocellular carcinoma: current progress and future directions*. Hepatology, 2014. **60**(5): p. 1776-82.
234. Kudo, M., *Immune Checkpoint Blockade in Hepatocellular Carcinoma: 2017 Update*. Liver Cancer, 2016. **6**(1): p. 1-12.
235. Prieto, J., I. Melero, and B. Sangro, *Immunological landscape and immunotherapy of hepatocellular carcinoma*. Nat Rev Gastroenterol Hepatol, 2015. **12**(12): p. 681-700.

236. Rehermann, B. and M. Nascimbeni, *Immunology of hepatitis B virus and hepatitis C virus infection*. Nat Rev Immunol, 2005. **5**(3): p. 215-29.
237. Guo, L., et al., *PD-L1 expression and CD274 gene alteration in triple-negative breast cancer: implication for prognostic biomarker*. Springerplus, 2016. **5**(1): p. 805.
238. Hodi, F.S., et al., *CTLA-4 blockade with ipilimumab induces significant clinical benefit in a female with melanoma metastases to the CNS*. Nat Clin Pract Oncol, 2008. **5**(9): p. 557-61.
239. Chuk, M.K., et al., *FDA Approval Summary: Accelerated Approval of Pembrolizumab for Second-Line Treatment of Metastatic Melanoma*. Clin Cancer Res, 2017.
240. Mahoney, K.M., G.J. Freeman, and D.F. McDermott, *The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma*. Clinical therapeutics, 2015. **37**(4): p. 764-82.
241. El-Far, M., et al., *CD160 isoforms and regulation of CD4 and CD8 T-cell responses*. J Transl Med, 2014. **12**: p. 217.
242. Crespo, J., et al., *T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment*. Curr Opin Immunol, 2013. **25**(2): p. 214-21.
243. Kyi, C. and M.A. Postow, *Checkpoint blocking antibodies in cancer immunotherapy*. FEBS Lett, 2014. **588**(2): p. 368-76.
244. Patel, S.P. and R. Kurzrock, *PD-L1 Expression as a Predictive Biomarker in Cancer Immunotherapy*. Mol Cancer Ther, 2015. **14**(4): p. 847-56.
245. Brahmer, J.R., et al., *Safety and activity of anti-PD-L1 antibody in patients with advanced cancer*. The New England journal of medicine, 2012. **366**(26): p. 2455-65.
246. Hoshida, Y., et al., *Gene expression in fixed tissues and outcome in hepatocellular carcinoma*. N Engl J Med, 2008. **359**(19): p. 1995-2004.
247. Kumar, B.S., et al., *Rapid and simple method of photobleaching to reduce background autofluorescence in lung tissue sections*. Indian J Biochem Biophys, 2015. **52**(1): p. 107-10.
248. Chen, C.L., et al., *PD-L1 expression as a predictive biomarker for cytokine-induced killer cell immunotherapy in patients with hepatocellular carcinoma*. Oncoimmunology, 2016. **5**(7): p. e1176653.
249. Ouyang, Q., et al., *Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1)*. Exp Gerontol, 2003. **38**(8): p. 911-20.
250. Addeo, R., M. Caraglia, and G. Iuliano, *Pembrolizumab: the value of PDL1 biomarker in head and neck cancer*. Expert Opin Biol Ther, 2016. **16**(9): p. 1075-8.
251. Aguiar, P.N., Jr., et al., *The role of PD-L1 expression as a predictive biomarker in advanced non-small-cell lung cancer: a network meta-analysis*. Immunotherapy, 2016. **8**(4): p. 479-88.
252. Li, T., C. Mello-Thoms, and P.C. Brennan, *Descriptive epidemiology of breast cancer in China: incidence, mortality, survival and prevalence*. Breast Cancer Res Treat, 2016. **159**(3): p. 395-406.
253. Yan, X., et al., *Incidence, mortality and survival of female breast cancer during 2003-2011 in Jiangsu province, China*. Chin J Cancer Res, 2016. **28**(3): p. 321-9.

254. Chen, K., et al., *[Incidence, mortality and survival rates of female breast cancer in Tianjin, China]*. Zhonghua Zhong Liu Za Zhi, 2002. **24**(6): p. 573-5.
255. Xing, Y., et al., *Survival analysis of patients with unilateral and bilateral primary breast cancer in Northeast China*. Breast Cancer, 2015. **22**(5): p. 536-43.
256. Galffy, G. and R. Puskas, *[Role of pembrolizumab in the treatment of non-small cell lung cancer (NSCLC)]*. Magy Onkol, 2017. **61**(1): p. 107-110.
257. Topalian, S.L., et al., *Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy*. Nat Rev Cancer, 2016. **16**(5): p. 275-87.
258. Zhang, Z., et al., *Immunopathogenesis and prognostic immune markers of chronic hepatitis B virus infection*. J Gastroenterol Hepatol, 2012. **27**(2): p. 223-30.
259. Gao, B., S. Radaeva, and O. Park, *Liver natural killer and natural killer T cells: immunobiology and emerging roles in liver diseases*. J Leukoc Biol, 2009. **86**(3): p. 513-28.
260. Bataller, R. and D.A. Brenner, *Liver fibrosis*. J Clin Invest, 2005. **115**(2): p. 209-18.
261. Friedman, S.L., *Mechanisms of hepatic fibrogenesis*. Gastroenterology, 2008. **134**(6): p. 1655-69.
262. Radaeva, S., et al., *Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners*. Gastroenterology, 2006. **130**(2): p. 435-52.
263. Lauren A. Zenewicz, G.D.Y., David M. Valenzuela, Andrew J. Murphy, Margaret Karow, and Richard A. Flavell, *IL-22 but not IL-17 provides protection to hepatocytes during acute liver inflammation*. Immunity, 2007.
264. Peppas, D., et al., *Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion*. J Exp Med, 2013. **210**(1): p. 99-114.
265. Bruggner, R.V., et al., *Automated identification of stratifying signatures in cellular subpopulations*. Proc Natl Acad Sci U S A, 2014. **111**(26): p. E2770-7.
266. Bendall, S.C., et al., *Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum*. Science, 2011. **332**(6030): p. 687-96.
267. Bodenmiller, B., et al., *Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators*. Nat Biotechnol, 2012. **30**(9): p. 858-67.
268. Qiu, P., et al., *Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE*. Nat Biotechnol, 2011. **29**(10): p. 886-91.
269. Anchang, B., et al., *Visualization and cellular hierarchy inference of single-cell data using SPADE*. Nat Protoc, 2016. **11**(7): p. 1264-79.
270. Maaten, L.v.d., *Visualizing Data using t-SNE*. Journal of Machine Learning Research 2008.
271. Maaten, L.v.d., *Learning a Parametric Embedding by Preserving Local Structure*. 2009.

272. Maaten, L.v.d., *Accelerating t-SNE using Tree-Based Algorithms*. Journal of Machine Learning Research, 2014.
273. Utzschneider, D.T., et al., *T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion*. Nat Immunol, 2013. **14**(6): p. 603-10.
274. Balkwill, F.R., M. Capasso, and T. Hagemann, *The tumor microenvironment at a glance*. J Cell Sci, 2012. **125**(Pt 23): p. 5591-6.
275. Bao, B., et al., *Targeting CSCs within the tumor microenvironment for cancer therapy: a potential role of mesenchymal stem cells*. Expert Opin Ther Targets, 2012. **16**(10): p. 1041-54.
276. Joyce, J.A. and D.T. Fearon, *T cell exclusion, immune privilege, and the tumor microenvironment*. Science, 2015. **348**(6230): p. 74-80.
277. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis*. Nat Med, 2013. **19**(11): p. 1423-37.
278. Sidaway, P., *Bladder cancer: Pembrolizumab is superior to chemotherapy*. Nat Rev Urol, 2017.
279. Hammerman, P., *Primary and adaptive resistance to checkpoint blockade in lung cancer*. Journal of Thoracic Oncology, 2016.