LLT1-MEDIATED IMMUNOTHERAPY FOR HEPATOCELLULAR CARCINOMA

THESIS

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CHAPTER I

INTRODUCTION

1.1 Hepatocellular Carcinoma

Hepatocellular carcinoma is the most common type of liver cancer and fifth most common cause of cancer-related death. Overall five-year survival rates for HCC remain a bleak 20%, with this percentage dropping significantly when diagnosed in late stages of development[1]. The rate of new cases and death rate has continued to consistently rise since 1992 and is projected to steadily increase through 2030, emphasizing the need for preventative measures and more effective forms of treatment. The most common predisposing factors for HCC include viral infection by HBV or HCV, chronic liver disease, diabetes, excess alcohol consumption, obesity, and non-alcoholic steatohepatitis (NASH). HCC growth patterns can vary depending on predisposing factors and include nodular, massive, and infiltrative [2]. When an individual has liver disease in the form of fibrosis or cirrhosis, regular screenings for HCC are strongly recommended to catch early signs of tumor growth. Elevated alpha-fetoprotein levels are the most common sign of HCC with over 75% of patients presenting with this symptom[3]. Following alpha-fetoprotein readings, either a CT

scan or MRI is performed to confirm the presence of a liver tumor, with a biopsy required in certain cases.

HCC rates per 100,000 persons have been shown to vary depending on location as certain countries have higher rates of predisposing diseases. These at-risk locations include China, Taiwan, Hong Kong, and sub-Saharan Africa. Males have been identified as significantly more at-risk of acquiring HCC with only 20% of cases being female[4]. Studies have shown that prevalence of HCC increases with age as the average age of diagnosis is 60 years old[5]. This elderly age further complicates the treatment process as some patients may not be as receptive to the aggressive therapy methods recommended. Multiple molecular pathway modifications have been identified in HCC which impact cell growth and angiogenesis, apoptosis, the cell cycle, and cell proliferative properties[6]. As such, HCC has been characterized by several genetic regulator mutations with the most commonly noted including TP53, CTNNB1 and those in the TERT promoter[7].

1.2 Current treatment options for Hepatocellular Carcinoma

Treatment options for HCC patients vary depending on both the stage of cancer development and the state of the liver at the time of diagnosis. Early-stage treatment options are generally surgical and can include resection of the tumor nodules, liver transplantation or radiofrequency ablation[8]. Liver transplant and resection of tumor nodules during this stage of development are currently considered the only curative options and patients must meet the Milan criteria (1 lesion $\leq$ 5 cm. or 2-3 lesions $\leq$ 3 cm.) in order to qualify [9, 10]. Although not all patients with early-stage HCC meet these restrictive qualifications, individuals that fall within this category of localized tumors have the best prognosis with a 34%, 5-year survival rate.

Survival rates drop to 12% when diagnosed in intermediate stages of HCC development, with more locoregional treatments offered including TACE (trans-arterial chemoembolization), TARE (trans-arterial radioembolization) and radiation[11]. While prognosis of patients in intermediate stages of HCC development can vary drastically depending on comorbidities and tumor presentation, treatment has remained relatively restricted[12]. Recently, research demonstrating the effect of TACE on immune cell function has indicated that expression of PD-L1 is upregulated in patients following treatment[13]. This interesting correlation of immunosuppression in an already significantly suppressed microenvironment has called attention to the possibility of using TACE combined with systemic immunotherapy to enhance treatment and reduce risk of relapse[14]. Clinical trials testing the efficacy of using a CTLA-4 or PD-L1 blockade following TACE in intermediate stage HCC are currently underway (NCT03638141).

Once HCC has spread to distant tissues and is considered in advanced stages, 5-year survival rates drop to a dismal 3%. Treatment strategies in this stage focus primarily on systemic options such as multi-kinase inhibitors, immune checkpoint inhibitors and multi-faceted combination therapies in an attempt to address the adapting issue of drug resistance and immune suppression [15] [16]. There are several different TKIs (tyrosine kinase inhibitors) which have been approved for late-stage HCC including Sorafenib, Lenvatinib, Regorafenib, and Cabozantinib[17]. Sorafenib is the most used TKI which targets VEGFR (vascular endothelial growth factor receptor) and PDGFR (platelet derived growth factor receptor) and has been the standard of care for advanced HCC since 2007. Although sorafenib was the primary treatment for late-stage HCC between 2007 and 2016, the scope of this form of treatment has been limited, with many patients non-responsive or developing resistance over time[15]. Additionally, randomized phase III trials demonstrated that sorafenib is primarily effective in patients who had prior HBV

or HCV infection, indicating the population-specific receptiveness to this systemic therapy[18]. Atezolizumab (anti-PD-L1 monoclonal antibody) in combination with Bevacizumab (anti-VEGF) became FDA approved for late-stage HCC in 2020 in an effort to combat the multifaceted microenvironment[19]. Although this form of therapy is currently considered one of the most effective options, data shows it has limited efficacy with only 18 out of 336 patients showing a complete response during clinical trials.

1.3 The Hepatocellular Carcinoma Microenvironment

The pathophysiology of HCC is complicated as many patients present with multiple comorbidities at once, often beginning with viral infection by HCV or HBV and progressing to cirrhosis or advanced fibrosis [20]. The damaged and mutated state of the liver due to liver disease often results in immune cell dysfunction and an altered microenvironment, putting patients at high risk for HCC [21]. It is estimated that approximately 90% of HCC cases are the result of chronic liver disease due to prior HCV or HBV infection, emphasizing the need for accessible antiviral therapies to reduce the rate of HCC contraction[22]. While both HCV and HBV can ultimately cause cancer, HCC occurs most commonly due to HBV, a DNA derived virus capable of integrating into the host genome. This process of integration and subsequent effects have been identified as key players in progression to HCC by inducing chromosomal instability and mutagenesis of HCC-associated genes[23]. Once the liver has progressed to a cancerous state, the co-diseased state of liver disease and HCC combats immune recognition by oncogenic regulation and mutations which can cause upregulation of biomarkers involved in immunosuppressive effects[24].

In the healthy liver, immune cells are exposed regularly to microbial products and dietary particles through portal circulation and while some inflammation is required for normal metabolic function and tissue remodeling, failure to regulate immune responses to these antigens can lead to an inflamed liver state [25]. Consequently, overly primed and desensitized immune cells in the liver could unintentionally overlook invasion or cancer proliferation. Liver resident immune cells, therefore, play a major role not only in regulation of normal biological processes, but also in ensuring that dysfunction in the form of autoimmune disease or malignancies does not prevail [26]. When dysregulation occurs in the form of excess inflammation or inadequate immune responses, a diseased state can ensue [27]. Once the liver has become cancerous, a highly hypoxic environment is induced, causing secretion of immunosuppressive molecules including cytokines, and chemokines from hepatocellular cells, myeloid derived suppressor cells (MDSCs), immature dendritic cells (DCs), stromal cells, and tumor-associated macrophages (TAMs) [28]. This secretion of molecules can not only suppress resident immune cell activity, but also stimulate suppressor cell activation. Indoleamine 2,3-dioxygenase (IDO) is a major contributing immunoregulatory enzyme which is upregulated in fibroblasts of HCC patients by proinflammatory cytokines such as IFN-γ [29]. This secretion of IDO acts by stimulating the activation of T-regulatory (Treg) cells which directly suppress activity of both $CD8^+$ and $CD4^+$ T cells and secrete suppressive cytokines such as IL-10 and TGF-β. Secretion of these cytokines can inhibit production of both IFN-γ and TNF-α by NK cells and even downregulate the expression of activating ligands on the surface of the tumor, further suppressing the NK cell response [30]. Unsurprisingly, upregulation of NK cell inhibitory receptors has been identified as a major proponent of the HCC microenvironment and is correlated with a poorer prognosis in HCC patients [31].

1.4 Functional Relevance of Intrahepatic Natural Killer Cells

Studies have shown that there is a significantly higher concentration of certain immune cells in the liver than other organs including NK cells, natural killer T (NKT) cells and Kupffer cells, with NK cells specifically playing an important role during cancer proliferation [32]. While NK cells make up ~8% of circulating lymphocytes, they are a highly concentrated immune cell population in the liver comprising ~40-50% of intrahepatic lymphocytes [33]. Although research has indicated that NK cells possess memory capabilities, they are considered part of the innate immune system and play an important role as first responders to malignant or virally infected cells. NK cells can be divided into two primary subsets; $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$ with roughly 50/50 proportions in the liver [34]. While the $CD56^{dim}$ subset is generally considered to have heightened cytotoxic capabilities comparatively, the liver resident (lr)$CD56^{bright}$ subset has shown increased lytic properties when co-incubated with HCC cells [34, 35]. The predominant lr$CD56^{bright}$ NK cell subset can be identified by simultaneous expression of CD69 and CXCR6, but additional subgroups have been identified which express CD49a[36]. In addition to this location-dependent shift of toxicity, intrahepatic NK cells also possess slightly differing expression of adhesion-associated surface markers which allow them to remain tissue-resident. LrNK cells produce multiple types of effector molecules including TRAIL, granzyme K, IFN-γ, TNF-α, and GM-CSF implicating the potential cytotoxic capabilities, but functional characteristics have not been extensively studied[37].

While functionally similar in some respect to CD8+ T cells, NK cells differ based on both activation properties as well as surface expression of immune-cell specific biomarkers [38]. In contrast to T-cells which are antigen-specific, NK cell activity is controlled via an intricate balance

of activating and inhibitory receptor interactions [39]. Receptor and ligand expression can vary depending on the environment as well as inducing factors such as tumor-secreted cytokines and chemokines [40]. Receptors expressed on the surfaces of NK cells interact with ligands expressed on the surfaces of target cells including host tissues, immune cells, and foreign invaders. Upon interaction with a virally infected or cancerous cell, stimulation occurs via activating receptor interactions and the NK cell releases cytotoxic granules onto the target cell, causing apoptosis and ultimately cell death [41]. Another important mechanism of NK cell activation occurs via antibody-dependent cell mediated cytotoxicity, also known as ADCC. When a target cell or cancer is treated with a monoclonal antibody specific for a biomarker expressed, an NK cell expressing the CD16 receptor can bind the Fc region of the antibody, inducing cytolytic activity and subsequent apoptosis of the target cell. This mechanism of NK activation enhances treatment in two ways; first, it blocks inhibitory interactions from being delivered to the immune cell and second, directly stimulates apoptosis of the target. When an NK cell interacts with a healthy cell via inhibitory receptor interactions, suppressive signals are deployed, and the immune cell continues with circulation [41]. Unsurprisingly, cancer cells have adopted this mechanism of immune evasion by upregulating surface expression of markers involved in sending inhibitory signals.

Research showing the correlation between upregulated biomarker expression and poor cancer prognosis in HCC patients signifies the importance of targeting these elements to improve patient recovery [42], [43]. NK cell immunotherapies in the form of adoptive transfer, monoclonal antibodies and CAR-NKs are currently being studied for their safety and efficacy in multiple different types of cancer including ovarian cancer, breast cancer and small-cell lung cancer [44] [45, 46]. Adoptive transfer of allogeneic NK cells for HCC has reached clinical trials in China and

Taiwan (<NCT04162158>, <NCT02008929>) but there has not been extensive research on NK-associated markers in HCC, providing a novel avenue to explore [47]. Since HCC induces the upregulation of biomarkers involved in suppressive effects, identification of receptors mediating these responses remains a research topic of interest [48]. Recently, research delving into characterization of novel NK cell receptors has indicated that potentially several different inhibitory biomarkers are expressed in HCC including a CLEC2D family receptor called LLT1 (lectin-like transcript 1) and PD-L1 (CD274, B7 homolog 1). This is indicative that multiple receptor-ligand interactions could be occurring during HCC resulting in the multi-level immunosuppressive effects seen [49].

1.5 Lectin-Like Transcript 1 (LLT1)

LLT1 is a C-type lectin transmembrane molecule encoded on chromosome 12 of the human genome and chromosome 6 in mice[50]. While several isoforms of LLT have been identified, LLT isoform 1 is the only surface resident biomarker, with other forms expressed intracellularly. Additionally, it has been shown that LLT1 is expressed intracellularly in most cells including those that are nontumorigenic, but transports to the surface of cancer cells to interact in a suppressive fashion during cancer proliferation[51]. The mechanism of LLT1 transportation to the cell surface is not well understood, but it is estimated that stress signals mediate this process. Expression of LLT1 has also been identified on the surfaces of several immune cell subsets including macrophages, germinal center B cells, T cells, plasmacytoid dendritic cells and NK cells. Unlike other C-type lectin domains which recognize carbohydrates, LLT1 interacts via protein-protein interactions and recognizes the NK cell receptor, NKRP1A (KLRB1, CD161). When LLT1 is expressed on the surface of a cancer cell and interacts via NKRP1A, NK cell activity is suppressed,

and the immune cell is unable to effectively recognize and lyse the cancer cell. Our lab has identified LLT1 as upregulated on the surfaces of several cancer cells including Ewing Sarcoma, Triple Negative Breast Cancer (TNBC), B-ALL, A-ALL and prostate cancer[52, 53]. Research has shown that use of an anti-LLT1 monoclonal antibody blocking LLT1-NKRP1A interactions results in blockage of suppressive signals, subsequently resulting in more active NK cells capable of recognizing and lysing the cancer[53]. These findings illustrate the potential of LLT1 as a therapeutic marker in several different types of cancer. Future research investigating these processes *in-vivo* in a mouse model are important for determining the safety and efficacy of blocking LLT1-mediated suppression for enhanced cancer cell lysis.

Interestingly, expression of LLT1 has also been noted as upregulated on the surface of virally infected cells including EBV and HIV infected B cells, RSV-infected lung epithelium and importantly HBV-infected liver cells due to viral integration and replication[54]. As previously discussed, HBV remains one of the primary threats and predisposing agents of HCC and as HBV remains untreatable, targeting LLT1 could potentially improve patient prognosis in the context of both viral infection and cancer proliferation. Research exploring the relationship between survival rates and expression of LLT1 in HPV-negative Oropharyngeal Squamous Cell Carcinoma implicates it as a marker of tumor aggressiveness[55]. Conversely, elevated expression of LLT1 on tumor-infiltrating lymphocytes (TILs) was strongly associated with improved patient prognosis. This relationship illustrates the tissue-dependent relevance of LLT1 as a prognostic factor, but additional research is needed to determine functional characteristics when expressed on various immune cell subsets.

When expressed on the surface of NK cells, LLT1 has been indicated to induce IFN-γ secretion when an anti-LLT1 monoclonal antibody is used to block the LLT1 receptor [56]. While

the signaling mechanism of LLT1 is not fully understood, research exploring the effects of different pharmacological inhibitors on IFN-γ production when LLT1 is expressed on an NK cell and stimulated has implicated the importance of the Src-PTK, MEK/ERK and p38 MAPK signaling pathways[56]. While this secretion of IFN-γ has not been shown to increase cytotoxic capabilities of NK cells, it could play an anti-tumor role in activation and recruitment of additional immune cells to the site of cancer proliferation.

Although research has not been done on LLT1 in HCC at the protein level, RNA sequencing data acquired from the online gene database The Cancer Genome Atlas (TCGA) demonstrates that CLEC2D, the gene coding for LLT1, is significantly upregulated in cancerous tissues of HCC patients compared to noncancerous counterparts. Expression at the transcript level, however, is not always indicative of surface proteins interacting at the functional level. Previous research found that resting PBMCs (peripheral blood mononuclear cells) expressed low levels of LLT1 at the gene level, but this expression was not observed in protein form[57]. Collectively, these findings suggest the relevance of exploring LLT1 further in order to determine expression and functionally characterize this biomarker in HCC.

1.6 Programmed Death Ligand-1 (PD-L1)

PD-L1 (also known as B7 homolog 1) is a type 1 transmembrane glycoprotein encoded by CD274 in the human genome. This ligand has been identified as upregulated on HCC cells and plays a role in suppressing immune cell activity[58]. The receptor for PD-L1 is expressed on both NK and T-cells and PD-1-PD-L1 interactions inhibit activation, proliferation, and cytotoxic capabilities of these effector cells. A number of signaling pathways have been implicated to regulate the PD-1/PD-L1 axis during cancer including JAK/STAT3, PI3K/AKT, MAPK, and

NFκB[59]. In the context of NK cell activity, this axis exerts inhibitory effects through repressing the activation of PI3K/ERK signaling in NK cells[60]. Its role in HCC has been well characterized, with the approved immunotherapy Atezolizumab targeting PD-L1, and Pembrolizumab targeting its receptor, PD-1.

Interestingly, PD-1 expression has been shown as upregulated on the surfaces of NK cells in the HCC microenvironment. This upregulated expression can be at least partially implicated in the NK cell suppressive effects seen during HCC, but the majority of research has focused on T-cell related effects [61]. Research comparing the percentage of PD-1 NK cells in healthy individuals vs. HCC patients found that there were significantly more PD-1+ NK cells in patients with cancer, with ~15% of circulating NK cells showing expression of this receptor[62]. The authors went on to block the PD-1 receptor via an anti-PD-1 monoclonal antibody Ab and found that this blockage significantly upregulated CD107a and IFN-γ production by these NK cells. This study demonstrated that while expression of PD-1 is typically correlated with T-cell suppressive effects, this inhibitory receptor is also capable of downregulating NK cell activity during HCC, allowing cancer cell evasion. While both anti-PD-1 and anti-PD-L1 monoclonal antibody therapies have been shown to increase the lifespan of recipients, there has been limited overall recovery when utilized as a monotherapy [63]. Currently, Atezolizumab (anti-PD-L1) is actively used in conjunction with an anti-VEGF (vascular endothelial growth factor) for advanced HCC patients and is considered the most effective late-stage immunotherapy available but has shown low success rates [64]. With a limited number of targets for ICI therapy in HCC, research focused on identification of potential targets is critical for improved treatment modalities.

1.7 Rationale for Combination Immunotherapy for Hepatocellular Carcinoma

Combining cancer treatments such as chemotherapy and radiation or chemotherapy and immunotherapy for enhanced targeting is not a new concept. Tumor resistance remains a major obstacle to treatment effectiveness, and researchers are finding that combating this resistance by targeting multiple different microenvironment characteristics could prove beneficial for enhanced tumor lysis. Recently, progress in combination therapies for HCC utilizing immune checkpoint inhibitors such as Atezolizumab with anti-angiogenesis factors have significantly improved patient prognosis with a 27% response rate compared to a 12% response rate in patients treated only with sorafenib[65]. While this response rate was promising as it more than double that of the prior first-line immunotherapy for HCC, 95% of the patients experienced side effects with 25% experiencing effects severe enough to discontinue treatment. Although progress, this clinical trial exemplifies the relevance of further exploring more efficacious and potentially less toxic alternative combination therapies. Recent studies exploring the functional properties of using a tumor-suppressing lipid molecule called nanoliposome C6-cermaide (which reduces the number of TAMs or tumor-associated macrophages) with CTLA-4 showed significantly higher suppression of tumor growth in a mouse model of HCC compared to monotherapy treatment[66].

The role of PD-L1 in suppressing $CD8^+$ T cell activity is well understood but targeting this ligand independently has shown limited success. Combining targeted immunotherapy against cancer-associated ligands LLT1 and PD-L1 could prove beneficial because it would provide a form of therapy that specifically upregulates both NK and $CD8^+$ T cell activity to overcome the microenvironment. Additionally, since many HCC patients co-present with both HBV and HCC, targeting LLT1 could prove synergistically efficacious by targeting both cancerous and virally infected cells.

1.8 Hypothesis and Specific Aims

LLT1 and PD-L1 are upregulated in hepatocellular carcinoma and this upregulation suppresses NK cell cytotoxic activity. Blocking LLT1 and PD-L1 with anti-LLT1 and anti-PD-L1 monoclonal antibodies will result in enhanced NK-cell mediated clearance of hepatocellular carcinoma cells.

Specific Aim I: Determine expression of LLT1 and PD-L1 in hepatocellular carcinoma cell lines compared to non-tumorigenic hepatocytes.

Specific Aim II: Determine NK cell cytolytic activity against HCC cells when using combination therapy of anti-LLT1 and anti-PD-L1 monoclonal antibodies.

Figure 1.1: **LLT1-Mediated Immunotherapy**. *Image borrowed from Sun et. al, 2019[67]*. Activation of NK cell using anti-LLT1 monoclonal antibody. (Left) Prostate and triple-negative breast cancer cells express LLT1 which can bind to NKRP1A on NK cells leading to inhibition of NK cell function. (Right) However, in the presence of anti-LLT1 antibodies, NK cells can be activated via two mechanisms: (1) blocking of LLT1 prevents inhibitory signal transmission; and (2) activation when NK cell receptor CD16 binds to anti-LLT1 antibody bound to LLT1 on cancer cells.

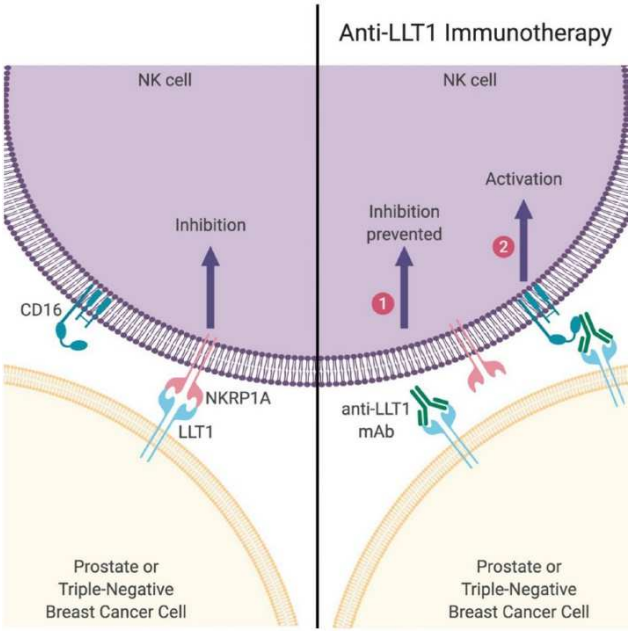


Figure 1.2: Proposed signaling pathway of LLT1-mediated IFN-γ production. *Image borrowed from Bambard et al., 2010[68]*. Probable signaling mechanism of lectin-like transcript-1-mediated (LLT1) IFN-γ production. IFN-γ inhibition data indicates Src-PTK, MEK/ERK and p38 MAPK are required for LLT1-induced IFN-γ production but PI3K, PKC and calcineurin/NF-AT are not required. Phosphorylation assays further indicate that ERK is employed by LLT1. IFN-γ transcriptional analysis indicates LLT1 does not alter IFN-γ transcription, suggesting post-transcriptional regulation is the mechanism by which LLT1 stimulated IFN-γ production. We suggest that LLT1 modulates IFN-γ post-transcriptional regulation via the MEK/ERK pathway and possibly via the p38 MAPK pathway working independent of PI3K.

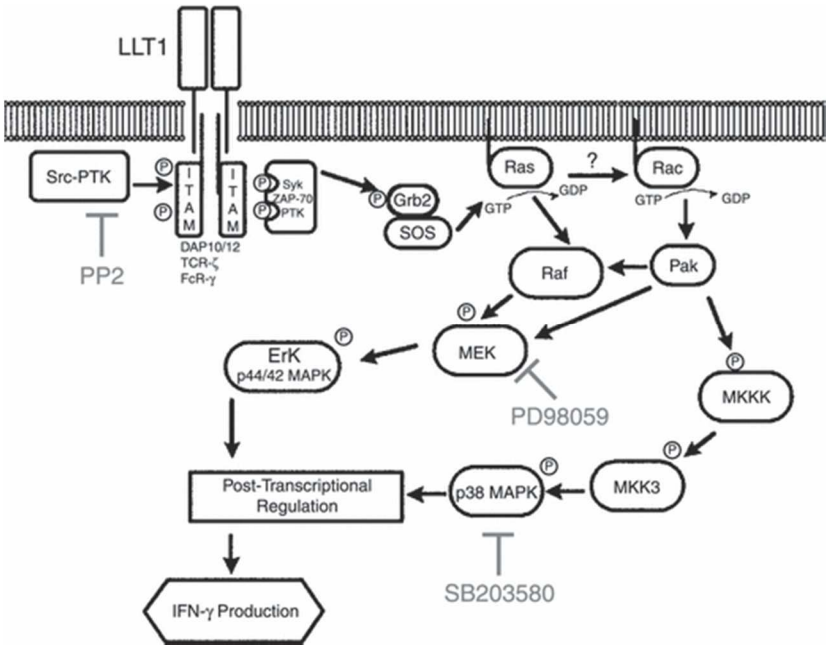
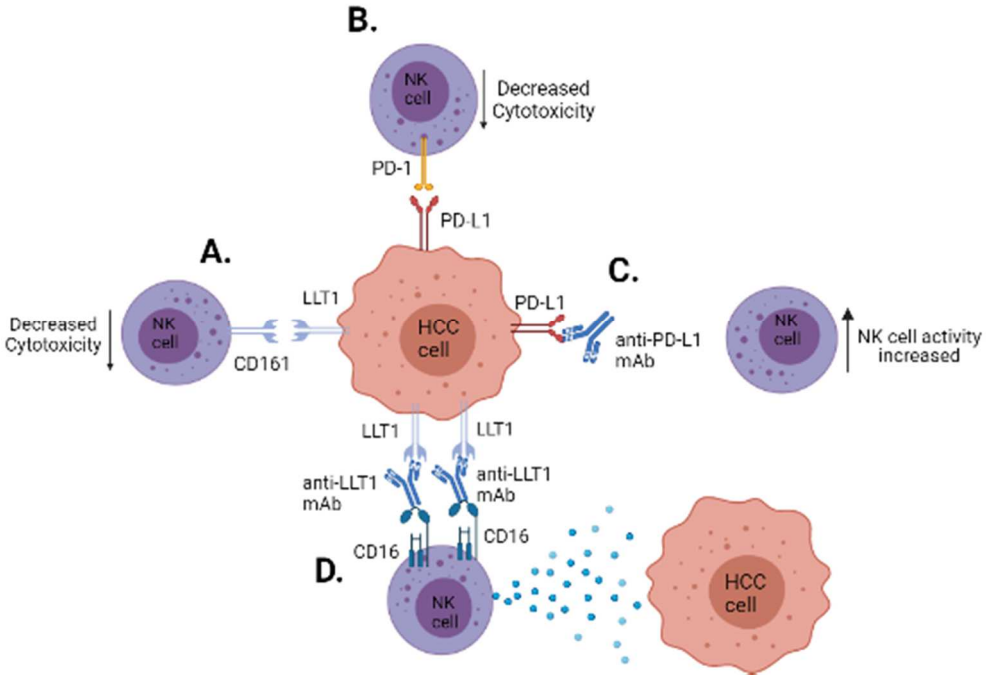


Figure 4.1 A visual representation of the effects of LLT1 and PD-L1 in HCC. A) LLT1 expressed on the surface of HCC interacts with CD161 (NKRP1A) expressed on the surface of an NK cell resulting in decreased NK-mediated cytotoxicity. B) PD-L1 expressed on the surface of HCC interacts via its ligand PD-1 to inhibit NK cell activity. C) Treatment of HCC with anti-PD-L1 monoclonal antibody increases NK cell activity. D) Anti-LLT1 monoclonal antibody treatment blocks LLT1-CD161 interactions. Circulating NK cells recognize the Fc region of the monoclonal antibody and bind via their CD16 receptors to induce ADCC (antibody-dependent cell mediated cytotoxicity), causing apoptosis of the target cancer cell. NOTE: ADCC induces apoptosis of the target cell it interacts with, the NK cell releasing lytic granules onto an independent HCC cell is shown to give a visual representation of this process. Image created in BioRender.



CHAPTER II

MATERIALS AND METHODS

2.1 Cell lines and Cell Culture

Experiments were conducted using four different HCC cell lines (Hep3B, HepG2, HUH7.5.1, SK-Hep1), control cells comprised of a prostate cancer cell line (DU145), a T-lymphoblast cell line (Jurkat), and a non-tumorigenic hepatocyte cell line (THLE2). All cells were maintained in a 6% CO2 incubator at 37 degrees Celsius and experiments were performed once cells reached 85-90% confluency. HCC cells were cultured using DMEM + 10% FBS +

antibiotic/antimycotic. Jurkat and DU145 cells were used as negative and positive controls respectively for LLT1 and were cultured using RPMI+10% FBS+antibiotic/antimycotic. Primary NK cells isolated from the peripheral blood were used for cytotoxicity assays and were cultured using 4+RPMI + 15% FBS+antibiotic/antimycotic. To minimize damage to surface receptors, a combination of 1X PBS + 1 mM EDTA was used in lieu of trypsin to release the adherent cells from the bottom of the flask. Cells were split once they reached ~90% confluency, generally 2-3 times per week, and new cell lines were thawed every 3-4 months depending on morphological characteristics and growth patterns.

2.2 RNA Sequencing Data (TCGA)

RNA sequencing data from HCC patients was obtained from The Cancer Genome Atlas (TCGA) to determine gene expression of LLT1 in tumorigenic vs. non-tumorigenic tissues. Data was transformed and compiled to generate tables and determine differences in expression between the various populations shown. Statistical analysis was performed using Prism and included a t-test and Tukey's multiple comparisons. A p-value<0.05 was considered significant.

2.3 Quantitative PCR (qPCR)

qPCR was performed to observe expression of CLEC2D (the gene coding for LLT1) and CD274 (the gene coding for PD-L1) via RNA extraction → cDNA synthesis → qPCR. Extraction was done using Trizol reagent and cDNA synthesis was performed using a high-capacity cDNA reverse transcriptase kit with RNase inhibitor. Following cDNA synthesis, nanodrop concentrations were recorded using a spectrophotometer and regular PCR was conducted using β-

actin primers to confirm that cDNA for each of the cell lines was present and in good quality. Once cDNA quality was confirmed, qPCR was conducted using TaqMan gene expression assays for CLEC2D and CD274. GAPDH was used as the reference gene for each cell line and FAM-MGB served as the probe, with FAM as the 5' reporter and MGB serving as the 3' quencher for maximal specificity. The TaqMan master mix contained the reference dye ROX to account for any background noise. Each sample was run in triplicates. Fold changes in gene expression were calculated using the ΔΔCT method. Statistical analysis included a One-way ANOVA to compare relative changes in gene expression of PD-L1 and LLT1 in HCC cells vs. non-tumorigenic hepatocytes. A p-value < 0.05 was considered significant.

2.4 Western Blot

Western blot analysis was performed to show total protein expression of both LLT1 and PD-L1 in HCC vs. non-tumorigenic hepatocytes. Antibodies used against LLT1 and PDL1 (AF3480, MAB1561) were ordered from R&D Systems. The LLT1 antibody was anti-human goat while the PD-L1 antibody was anti-human mouse. A donkey anti-goat HRP antibody was used against the primary LLT1 antibody for detection while a goat anti-mouse HRP antibody was used against the primary PD-L1 antibody. β-actin was used as a loading control and image was analyzed using ImageJ software. Relative densities are shown in bar graphs to compare differences.

2.6 Flow Cytometry

Flow cytometry was performed to determine cell surface expression of LLT1 and PD-L1 in cell lines. Anti-LLT1 and anti-PD-L1 mouse anti-human antibodies conjugated to phycoerythrin

(PE) were used to detect surface expression of LLT1 and PD-L1. Antibodies for flow cytometry (FAB3480P, FAB1561P-025) were ordered from R&D Systems. Since both antibodies were conjugated to PE, surface expression of LLT1 and PD-L1 was determined by staining separate cell samples taken from the same flask. An isotype control (mIgG1-PE) was used for each cell line to account for any potential non-specific antibody binding and DU145 was used as a positive control. Analysis of samples was done using FlowJo software. Mean fluorescent intensity ratios (MFIRs) were calculated as the ratio of experimental live cells positive for PE to the number of live isotype control cells positive for PE. An MFIR of >1.00 indicates expression of LLT1 or PD-L1, while an MFIR of less than or equal to 1.00 indicates no expression compared to the isotype control. Statistical analysis of MFIRs included one-way ANOVA to compare the expression of LLT1 and PD-L1 in HCC cells to non-tumorigenic hepatocytes. Graphs illustrating the percent of the cell populations positive for LLT1 and PD-L1 were generated by subtracting the percent of cells positive in the isotype control in each sample from the experimental sample specific for LLT1 or PD-L1. Statistical analysis included one-way ANOVA to compare the percentage of cells positive in the non-tumorigenic hepatocytes to the percentage positive in the HCC cells.

2.7 Confocal Microscopy

Confocal microscopy was performed to determine localized expression of LLT1 and PD-L1 in cell lines. Antibodies were ordered from R&D Systems (AF3480, MAB1561). Primary anti-human anti-PD-L1 antibody is sourced from a monoclonal mouse IgG1 and anti-human anti-LLT1 antibody is sourced from a polyclonal goat IgG. Respective isotype controls (mIgG1) were used for LLT1 and PD-L1 to account for background noise. The secondary antibody used for LLT1 is a donkey anti-goat Alexa Fluor-488 antibody. The secondary antibody used for PD-L1 is a goat

anti-mouse FITC antibody. Coverslips were coated with Poly-L lysine and 20,000 cells were grown overnight on coverslips. 2-4% paraformaldehyde was used to fix the cells prior to beginning the staining process. Blocking buffer was used which contained 5-10% serum against the secondary antibody host species. Primary staining was done, and dilutions contained serum against the secondary antibody to reduce non-specific binding. Cells were placed in 4 degrees Celsius fridge in a mock moist chamber overnight. The following day, secondary staining was performed for 1 hour at room temperature, followed by counterstaining with DAPI. Coverslips were then mounted on slides using ProLong Gold Anti-Fade Mountant and sealed using clear nail polish. Slides were imaged using an Inverted LSM 880 Airyscan confocal imager. Z-stack images were taken on 20X. Images generated were normalized to isotype control coverslips made for each cell line to account for any potential non-specific binding. ImageJ software was used for image analysis and channels were split to show individual staining of Alexa-Fluor 488 or FITC, DAPI and a composite.

2.8 PBMC and NK Cell Isolation

Whole blood was harvested from a donor using ACD tubes containing anticoagulant. Lymphoprep was added to a Leucosep tube for the PBMC isolation and whole blood carefully added. Tubes were spun at 3000x g to obtain separation of phases illustrating the plasma, RBCs, lymphoprep and thin layer of PBMCs. The PBMC layer was collected and washed several times followed by counting with a hemacytometer. Once the PBMC count was established, a Miltenyi Biotec NK cell isolation kit (guaranteed 98%+ purity) was utilized to separate the NK cell subset from other immune cells. Finally, the cell suspension was applied to an LS column and the flow-through containing pure NK cells was collected. Following collection, NK cell counts were

obtained, and NK cells placed in a 6% CO2, 37 degrees Celsius incubator in a T-25 flask with 4+RPMI+15% FBS+An/An. NK cells were not expanded using cytokines, so functional assays were performed within 24 hours of the collection to ensure activity.

2.9 Cytotoxicity Assays

Cytotoxicity experiments were conducted using a Delfia assay kit from PerkinElmer. The effector (primary NK cells) to target (cell lines) E:T ratios were 25:1, 5:1 and 1:1 with a control and 3 separate experimental groups for each of the 4 cell lines. The control was treated with a non-specific monoclonal antibody (mIgG1), E1 (experimental group 1) was treated with anti-LLT1 monoclonal antibodies, E2 (experimental group 2) was treated with anti-PD-L1 monoclonal antibodies and E3 (experimental group 3) was treated with a combination of anti-LLT1 and anti-PD-L1 monoclonal antibodies. Following antibody treatment, cells were infused with BATDA reagent. Upon co-incubation of primary NK cells with the treated cell lines, the plate was placed in a 37 degrees C, 6% CO2 incubator for 2 hours. The plate was then centrifuged for 5 minutes at 500 x g to pellet the cells and 20 uL of the supernatant was collected and treated with Europium solution. Percent lysis was measured using a plate reader and calculated based on the level of fluorescence detected in the supernatant.

CHAPTER III

RESULTS

Specific Aim I – Determine expression of LLT1 and PD-L1 in hepatocellular carcinoma cell lines compared to non-tumorigenic hepatocytes.

Rationale for Specific Aim 1

Current therapeutic options for HCC patients remain minimally successful, especially in late stages of cancer development. With previously explored options targeting angiogenesis factors and focused on mediating T-cell activity, recent exploration has indicated that HCC could be receptive to NK cell immunotherapeutic options[69]. As such, a clinical trial is currently underway

to observe the efficacy of using adoptive transfer of NK cells for late-stage HCC patients (NCT05040438). Novel immunotherapy research focused on identification of new markers that could serve as therapeutic targets is crucial for enhanced treatment that specifically lysis cancer cells. Expression of LLT1 has been shown as upregulated on the surface of multiple different types of cancer and anti-NK cell activity has been well characterized. As HCC should, in theory, provide an excellent target for NK-mediated immunotherapy, steps were taken to determine the expression of LLT1 in HCC to elucidate its therapeutic value.

3.1 LLT1 and PD-L1 are Upregulated in HCC Cells Compared to Non-tumorigenic Hepatocytes

3.1.1 LLT1 *Gene Expression Data in HCC patient tissue samples*

Gene expression data was obtained from the mass database The Cancer Genome Atlas (TCGA) to observe expression of LLT1 in cancerous tissues of HCC patients. In figure 3.1a, gene expression of LLT1 in 50 non-tumorigenic hepatocyte patient samples was compared to 371 HCC patient samples by RNA seq. analysis. Data showed that gene expression of LLT1 was significantly upregulated in the HCC patient samples with transcript abundance quantification of 7.6 ± 0.08 in non-tumorigenic tissue samples 8.27 ± 0.04 in HCC tissues. Statistical analysis demonstrated a $p\text{-value}<0.0001$.

To determine if this gene expression varied depending on stage of tumor development, data was compiled of HCC patients with tumor grades 1-4 and compared to gene expression in non-tumorigenic tissues and each consecutive grade. The non-tumorigenic tissue group was

represented by 50 patient samples, G1 (grade 1) was represented by 55 tissue samples, G2 was represented by 177 tissue samples, G3 by 122 and G4 by 12. The respective transcript abundance for each consecutive sample group was 7.6 ±0.08 (normal), 8.02±0.07 (G1), 8.2±0.05 (G2), 8.45±0.06 (G3) and 8.81±0.2 (G4). Tukey's multiple comparisons test comparing normal to cancerous tissues demonstrated significance between normal vs. G1 (p-value 0.017), normal vs. G2 (p-value<.0001), normal vs. G3 (p-value<.0001), normal vs. G4 (p-value<.0001). Significance was also noted between grades G1 vs. G3 (p-value 0.0015), G1 vs. G4 (p-value 0.003), G2 vs. G3 (p-value .02) and G2 vs. G4 (p-value 0.025). Significance was not observed between groups G1 vs. G2 (p-value 0.43) and G3 vs. G4 (p-value of 0.39).

Racial differences in gene expression levels of LLT1 in patients with HCC was also observed. Non-tumorigenic (normal) tissues were not divided based on race and was comprised of 50 patient samples, 185 Caucasian American samples, 17 African American samples and 159 Asian American samples. Significance was detected between normal (7.6±0.08) vs. each of the respective samples, as expected. Transcript abundance in Caucasian American individuals was noted as 8.16±0.05, in African Americans, 8.3±0.22 and in Asian Americans, 8.41±0.06. While no significance in transcript levels of LLT1 was noted between Caucasian American tissues and African American tissues or African American vs. Asian American, there was significant difference in gene expression detected between Caucasian American and Asian American patient samples demonstrated by a p-value of 0.004.

To determine if gene expression of LLT1 is correlated with percent survival of HCC patients, data was put into a line plot to demonstrate survival rate based on low/medium or high expression of LLT1. Graphs were separated based on tumor grade of 1+2 and 3+4. Results did not indicate a statistical significance between survival rate and low/medium and high expression of

LLT1 in HCC patients with tumor grade 1+2. A strong correlation was noted, however, in tumor grade 3+4 with increased expression of LLT1 a prognostic indicator of decreased percent survival (p-value of 0.017).

3.1.2 *mRNA Expression of LLT1 in HCC Cell Lines*

To determine if mRNA expression of LLT1 is upregulated in HCC cell lines compared to non-tumorigenic hepatocyte cells, qPCR was performed using TaqMan reagents. THLE2 (nontumorigenic hepatocytes) were normalized to one and everything higher than 1 was considered upregulated gene expression comparatively. Gene expression data was analyzed using the ΔΔCT method. Statistical analysis included one-way ANOVA to compare mRNA expression of LLT1 in non-tumorigenic hepatocytes to HCC cell lines. mRNA expression of LLT1 in HepG2, Hep3B, HUH7.5.1, and SK-Hep1 was shown as upregulated by 22%, 28%, 257%, and 293% compared to the non-tumorigenic hepatocytes, respectively. Statistical analysis demonstrated a significance in mRNA expression in THLE2 compared to HUH7.5.1 (p-value 0.0002) and SK-Hep1 (p-value 0.0001). Since a combination immunotherapy will be investigated for efficacy targeting both LLT1 and PD-L1, gene expression of PD-L1 in non-tumorigenic hepatocytes was compared to HCC cell lines. Interestingly, results showed that gene expression of PD-L1 was downregulated in all HCC cell lines compared to non-tumorigenic cells at p-values < 0.0001. While surprising considering PD-L1 is not typically reported as expressed on healthy cells, gene expression is not always directly translatable and correlated to surface expression.

3.1.3 *Total Protein Expression of LLT1 and PD-L1 in HCC Cell Lines*

Western blot was performed to look at total protein expression of both LLT1 and PD-L1 in HCC cell lines compared to non-tumorigenic hepatocytes. Western blot showed that expression of LLT1 in THLE2 (non-tumorigenic hepatocytes) was lower than in HCC cell lines. Conversely, total protein expression of PD-L1 was upregulated in THLE2 and SK-Hep1 cell lines and minimally expressed in Hep3B and HUH7.5.1 cell lines.

3.1.4 Cell *Surface Expression of LLT1 and PD-L1 in HCC Cell Lines*

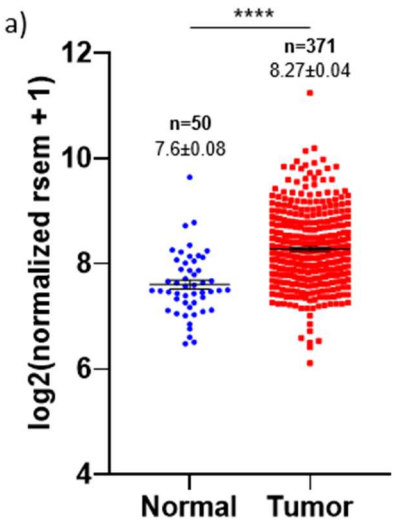
Flow cytometry was performed to look at cell surface expression of LLT1 and PD-L1 where it would be considered functionally relevant in the context of immunotherapeutic targeting. Flow cytometry staining of each ligand was done a minimum of 3 times per cell line. An isotype control was used as the negative control and used for each sample run and is represented by the blue histogram. Average MFIR ratios were calculated using FlowJo software and are the ratio of the fluorescent intensity of the experimental/negative control. An MFIR>1 indicates upregulated expression. The MFIR of LLT1 on the surface of THLE2 cells was averaged over multiple experiments and found to be 1.21. The average MFIR of LLT1 on Hep3B, HUH7.5.1, HepG2 and SK-Hep1 was found to be 4.09, 4.59, 1.81, and 4.24, respectively. One-way ANOVA indicated statistical significance between THLE2 and Hep3B (p-value<0.05), THLE2 and HUH7.5.1 (p-value<0.05), and THLE2 and SK-Hep1 (p-value<0.05), but a clear trend in increased expression on HCC cell lines is seen regarding all cell lines. Surface staining of PD-L1 showed an average MFIR on THLE2, Hep3B, HUH7.5.1, HepG2, and SK-Hep1 of 0.91, 2.45, 2.19, 1.77, and 1.88, respectively. While statistical significance was not detected between the non-tumorigenic and HCC cell lines regarding degree of PD-L1 expression, a clear trend demonstrates that PD-L1 is

upregulated on HCC cells compared to non-tumorigenic hepatocytes. The percentage of cells positive for LLT1 and PD-L1 was also observed using flow cytometry. Non-tumorigenic THLE2 cells had 4.55% cells that were detected as positive for LLT1 whereas Hep3B, HUH7.5.1, HepG2, and SK-Hep1 had 34.7%, 35.4%, 14.3%, and 35.6% positive cells respectively. One-way ANOVA was performed for statistical analysis and significance was detected between THLE2 and Hep3B, THLE2 and HUH7.5.1 and THLE2 and SK-Hep1 cells. Although HepG2 cells presented with a higher population of positive cells, significance was not detected. For PD-L1 staining, 5.9% of non-tumorigenic THLE2 cells were positive, whereas Hep3B, HUH7.5.1, HepG2, and SK-Hep1 had 45.5%, 31.7%, 22.8% and 30.08% that were positive for PD-L1 respectively. One-way ANOVA analysis indicated significance between THLE2 and Hep3B cell lines only, but a trend in increased expression of PD-L1 on HCC cells was noted.

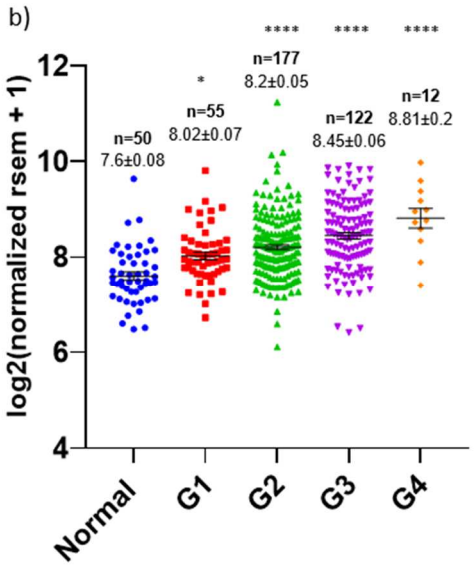
3.1.5 *Localized Cell Surface Expression of LLT1 and PD-L1 in HCC Cell Lines*

Confocal microscopy staining was done to show localized, qualitative surface expression of LLT1 on HCC cells. Surface staining for LLT1 on the surface of cell lines showed an interesting distribution of the ligand. For SK-Hep1 and HepG2, expression appeared to form the characteristic ring generally detected, however, HUH7.5.1 and Hep3B cells demonstrated almost clustered expression on the surface. Respective negative (mIgG1 isotype controls) were included for each cell line and treated in an identical fashion to account for any potential background fluorescence.

Figure 3.1: HCC patients exhibit upregulated gene expression of LLT1. RNA sequencing data from TCGA was acquired showing gene expression of LLT1 in tumorigenic tissues of HCC patients compared with non-tumorigenic counterparts. Singular dots represent one patient sample and the number of individuals in each cohort is represented above each group. Median normalized expression (log2) is given along with standard deviation (±) of each data set. a) Gene expression of LLT1 in normal vs. tumor tissue of HCC patients. b) Gene expression of LLT1 in HCC patients with increasing tumor grade. c) Gene expression of LLT1 across racial groups; CA – Caucasian American, AA – African American, AS – Asian American. Tukey's multiple comparisons test was used for statistical analysis. * p-value<.05, ** p-value<.005, **** p-value<.0001.

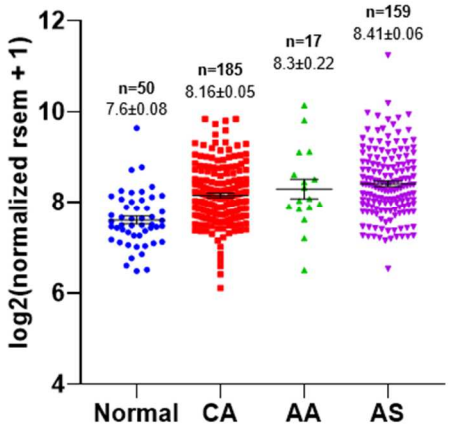

a)
****
n=371
8.27±0.04
n=50
7.6±0.08
log2(normalized rsem + 1)
12
10
8
6
4
Normal
Tumor

b)



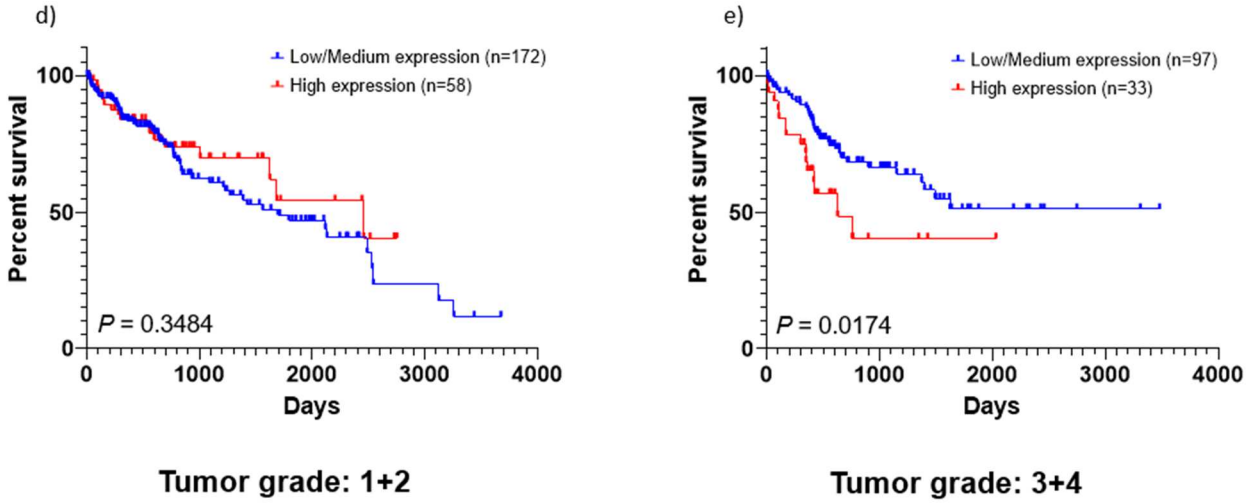
Tukey's multiple comparisons test	Summary	P Value
Normal vs. G1	*	0.0166
Normal vs. G2	****	<0.0001
Normal vs. G3	****	<0.0001
Normal vs. G4	****	<0.0001
G1 vs. G2	ns	0.4283
G1 vs. G3	**	0.0015
G1 vs. G4	**	0.0030
G2 vs. G3	*	0.0222
G2 vs. G4	*	0.0250
G3 vs. G4	ns	0.3946

c)



Tukey's multiple comparisons test	Summary	Adjusted P Value
Normal vs. CA	****	<0.0001
Normal vs. AA	**	0.0022
Normal vs. AS	****	<0.0001
CA vs. AA	ns	0.8528
CA vs. AS	**	0.0041
AA vs. AS	ns	0.9176

Figure 3.2: **HCC patient survival rates decrease with increased gene expression of LLT1 in stages 3+4 of tumor development.** These tables represent percent survival of HCC patients based on high or low gene expression of LLT1. Data was obtained and compiled from TCGA similarly to the prior figure. Each square represents a single patient in the data set. d) Percent survival of HCC patients with tumor grade of 1 or 2 based on low/medium or high expression of LLT1. e) Percent survival of HCC patients with tumor grade of 3 or 4 based on low/medium or high expression of LLT1.



Tumor grade: 1+2 **Tumor grade: 3+4**

Figure 3.3: **mRNA expression of LLT1 is upregulated in HCC cell lines.** Gene expression analysis was performed of LLT1 and PDL1 in HCC cell lines HepG2, Hep3B, HUH7.5.1 and SK-Hep1 compared to non-tumorigenic hepatocyte cell line, THLE2. Bars are representative of two independent experiments done in triplets. a) Gene expression of LLT1 in HCC cell compared to non-tumorigenic hepatocytes. b) Gene expression of PDL1 in HCC cell lines compared to non-tumorigenic hepatocytes. One-way ANOVA was used for statistical analysis and significance was considered p-value<.05. *** p-value<.001, ****p-value<.0001.

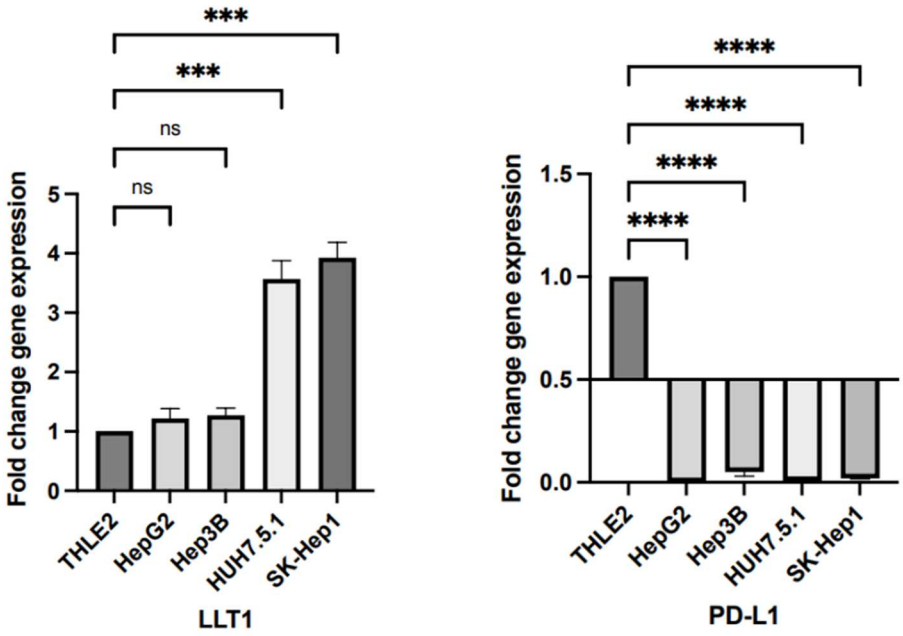


Figure 3.4: Total protein expression of LLT1 is upregulated in HCC cell lines. Western blot analysis was performed to observe total protein expression of both LLT1 and PD-L1 in HCC cell lines compared to non-tumorigenic hepatocytes. a) Western blot showing total protein expression of LLT1 and PDL1 in HCC cell lines Hep3B, HUH7.5.1 and SK-Hep1 vs. nontumorigenic hepatocytes, THLE2. Image was analyzed using ImageJ software. b) Bar graphs representative of relative expression of LLT1 and PDL1 in the designated cell lines over two independent experiments. Standard deviation bars represent the variation between the two experiments.

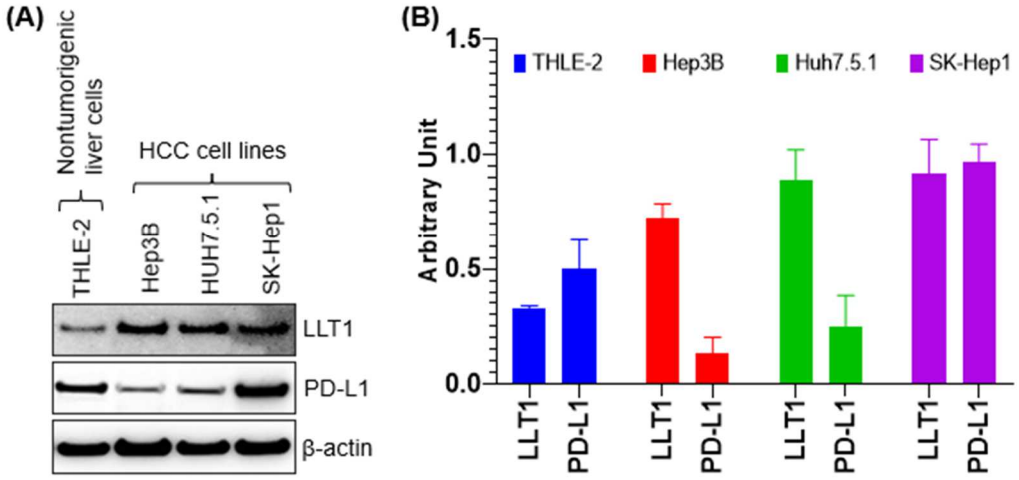
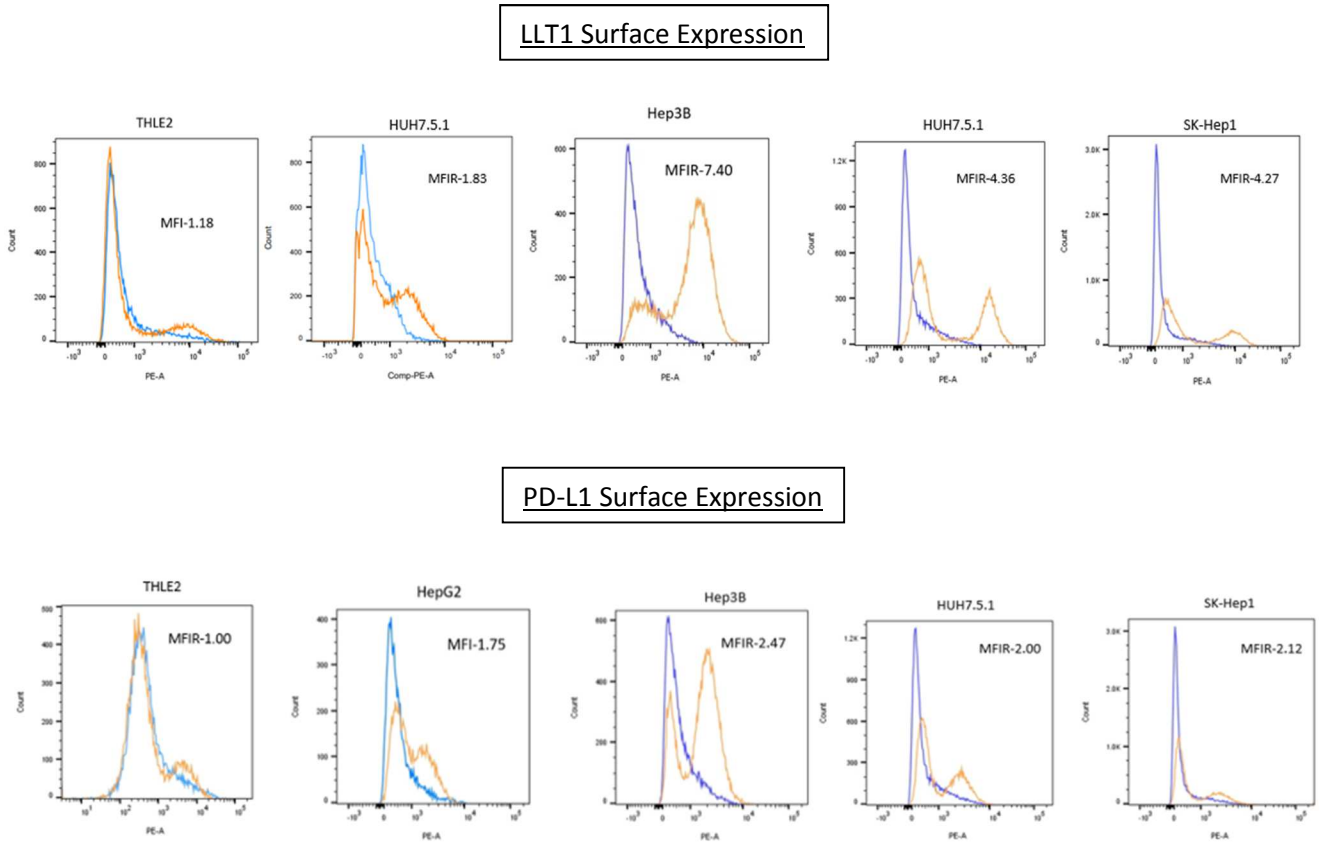
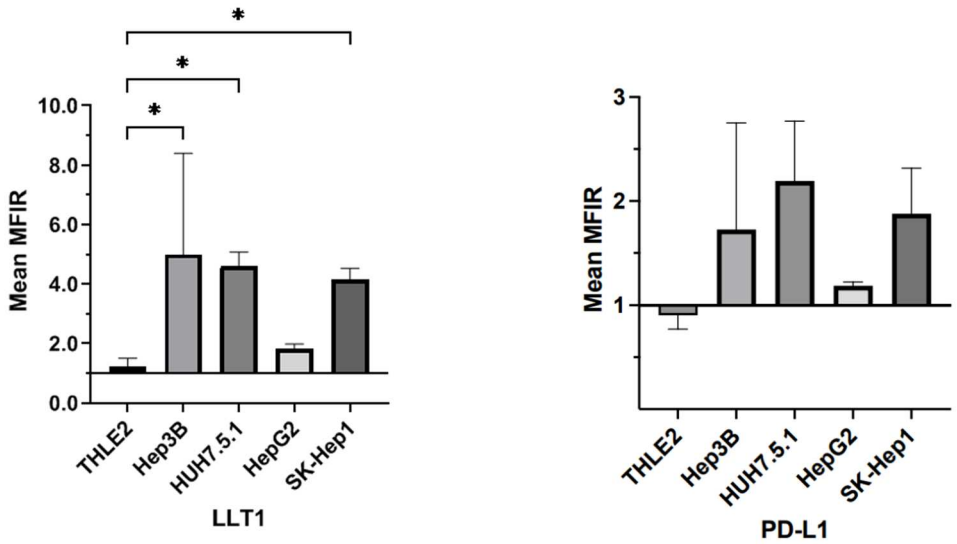


Figure 3.6: LLT1 and PD-L1 are upregulated on the surface of HCC cell lines. This figure demonstrates flow cytometry data showing surface expression of LLT1 and PDL1. The blue histogram represents the negative isotype control (mIgG1) used for each cell line. The orange histogram represents the target protein of interest, either LLT1 or PD-L1. A) The top row of histogram graphs shows surface expression of LLT1, and the bottom row shows surface expression of PDL1 in HCC cell lines Hep3B, HUH7.5.1, HepG2, SK-Hep1 compared to nontumorigenic hepatocytes, THLE2. B) Surface expression was measured 3+ independents times for each ligand and averaged. C) Graphs representing the percentage of cells that stained positive for LLT1 or PD-L1 in each experiment.

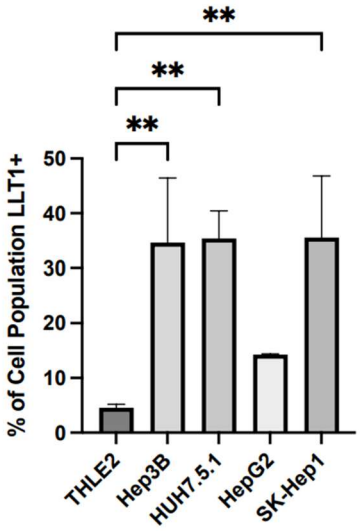
A)


LLT1 Surface Expression
THLE2
MFI-1.18
HUH7.5.1
MFIR-1.83
Hep3B
MFIR-7.40
HUH7.5.1
MFIR-4.36
SK-Hep1
MFIR-4.27
PD-L1 Surface Expression
THLE2
MFIR-1.00
HepG2
MFI-1.75
Hep3B
MFIR-2.47
HUH7.5.1
MFIR-2.00
SK-Hep1
MFIR-2.12

B)


Mean MFIR
10.0
8.0
6.0
4.0
2.0
0.0
THLE2
Hep3B
HUH7.5.1
HepG2
SK-Hep1
LLT1
Mean MFIR
3
2
1
THLE2
Hep3B
HUH7.5.1
HepG2
SK-Hep1
PD-L1

C)


**
**
**
% of Cell Population LLT1+
50
40
30
20
10
0
THLE2
Hep3B
HUH7.5.1
HepG2
SK-Hep1

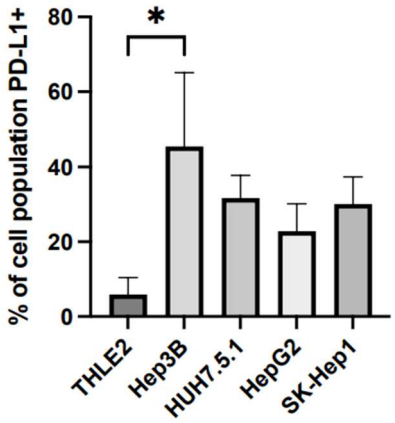
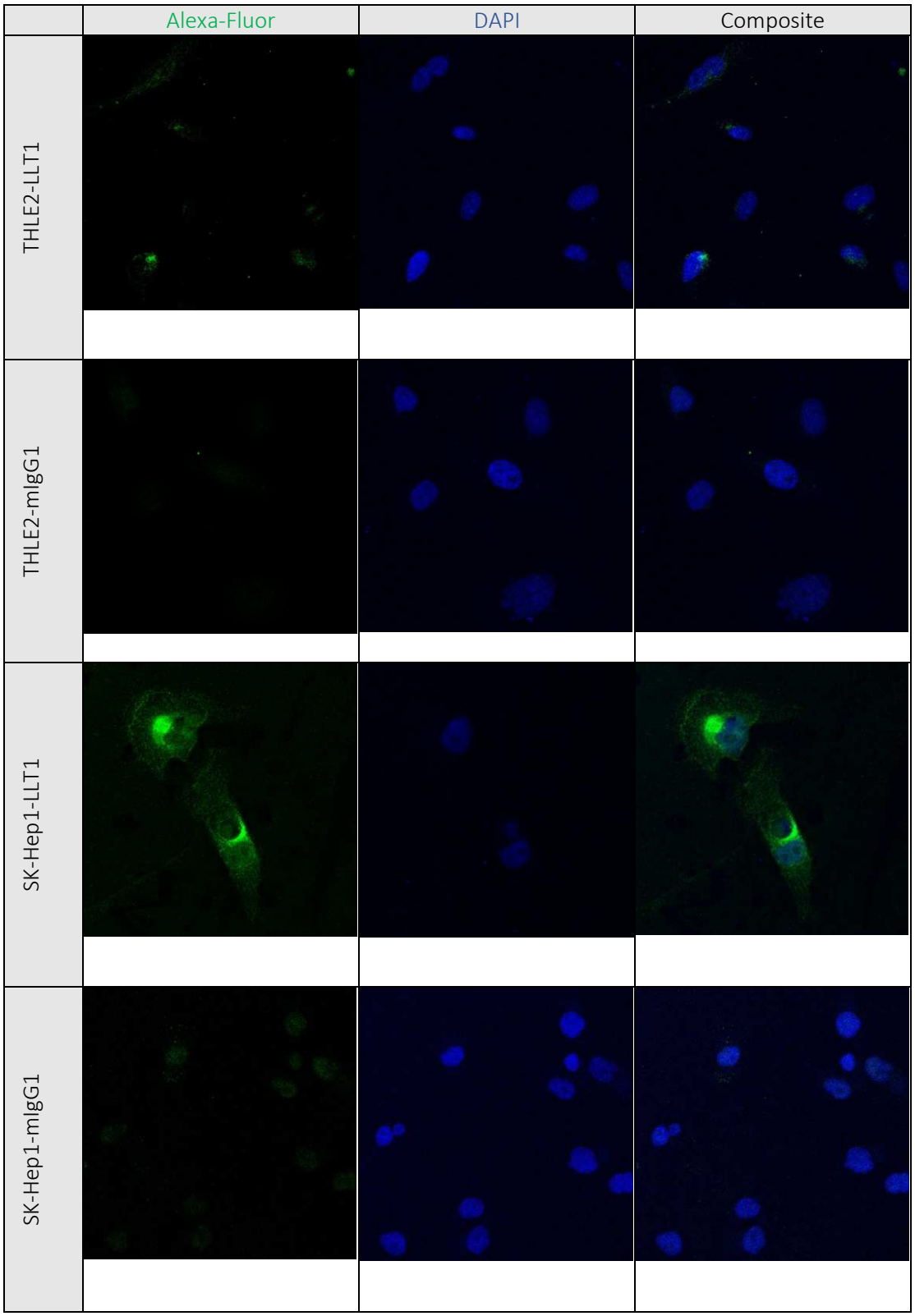
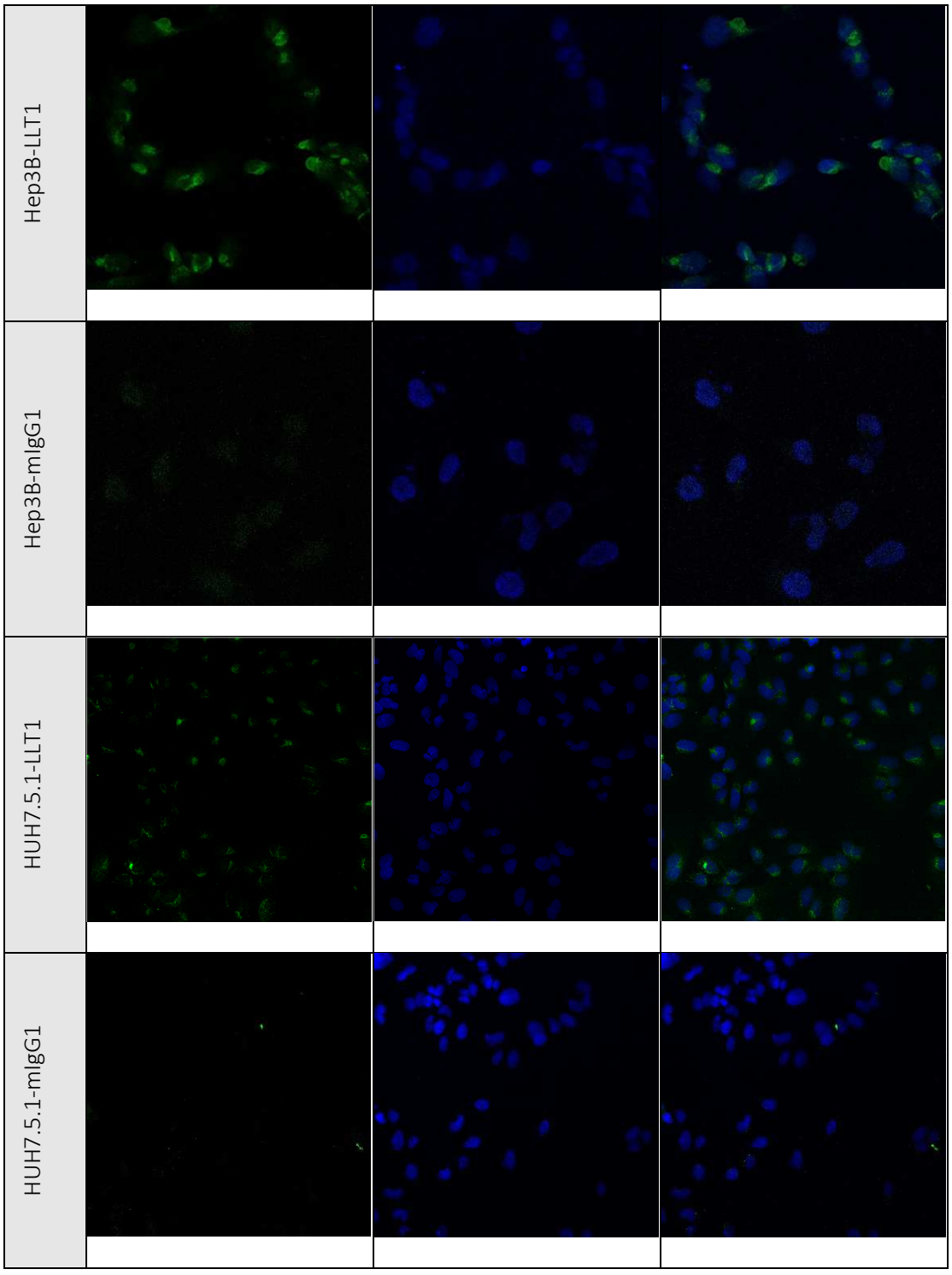

*
% of cell population PD-L1+
80
60
40
20
0
THLE2
Hep3B
HUH7.5.1
HepG2
SK-Hep1

Figure 3.7: LLT1 is localized at the cell surface in HCC cell lines. An isotype control (mIgG1 followed by secondary staining with Alexa-Fluor 488) was used for each of the cell lines to normalize Alexa-Fluor 488 intensity. Split channels/composites were generated using ImageJ software. Alexa-Fluor 488 staining represents expression of LLT1. Counterstaining with DAPI was done to show expression of LLT1 relative to the nucleus.


Alexa-Fluor
DAPI
Composite
THLE2-LLT1
THLE2-mIgG1
SK-Hep1-LLT1
SK-Hep1-mIgG1


Hep3B-LLT1
Hep3B-mIgG1
HUH7.5.1-LLT1
HUH7.5.1-mIgG1

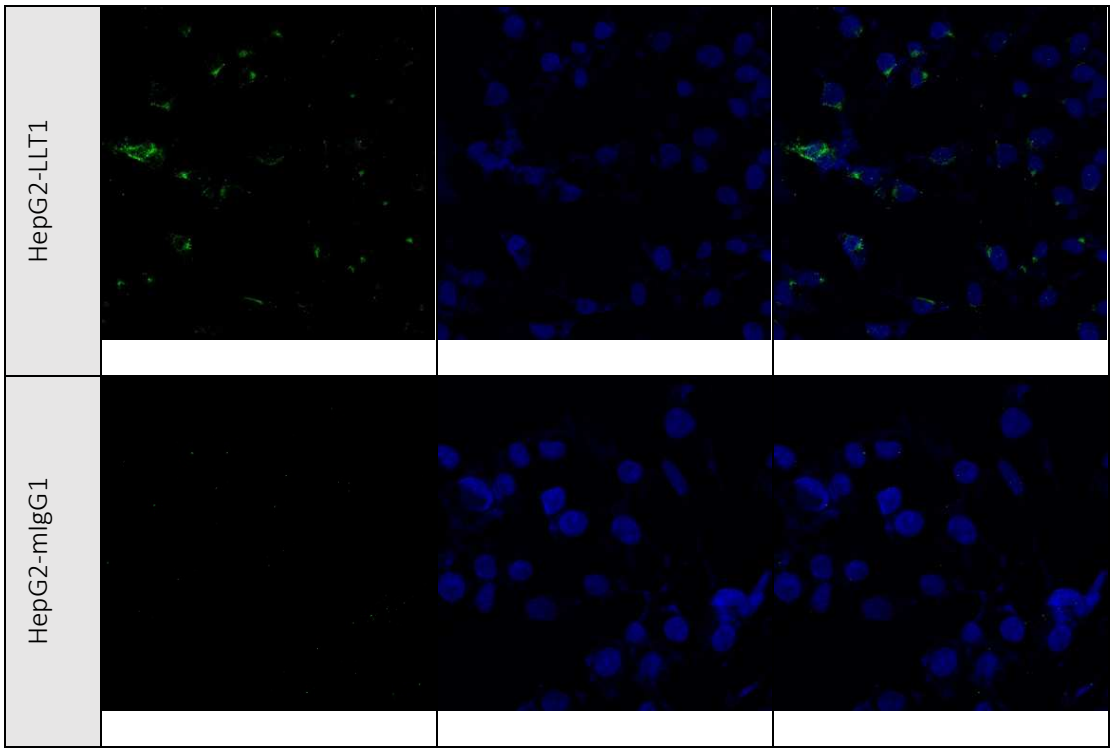
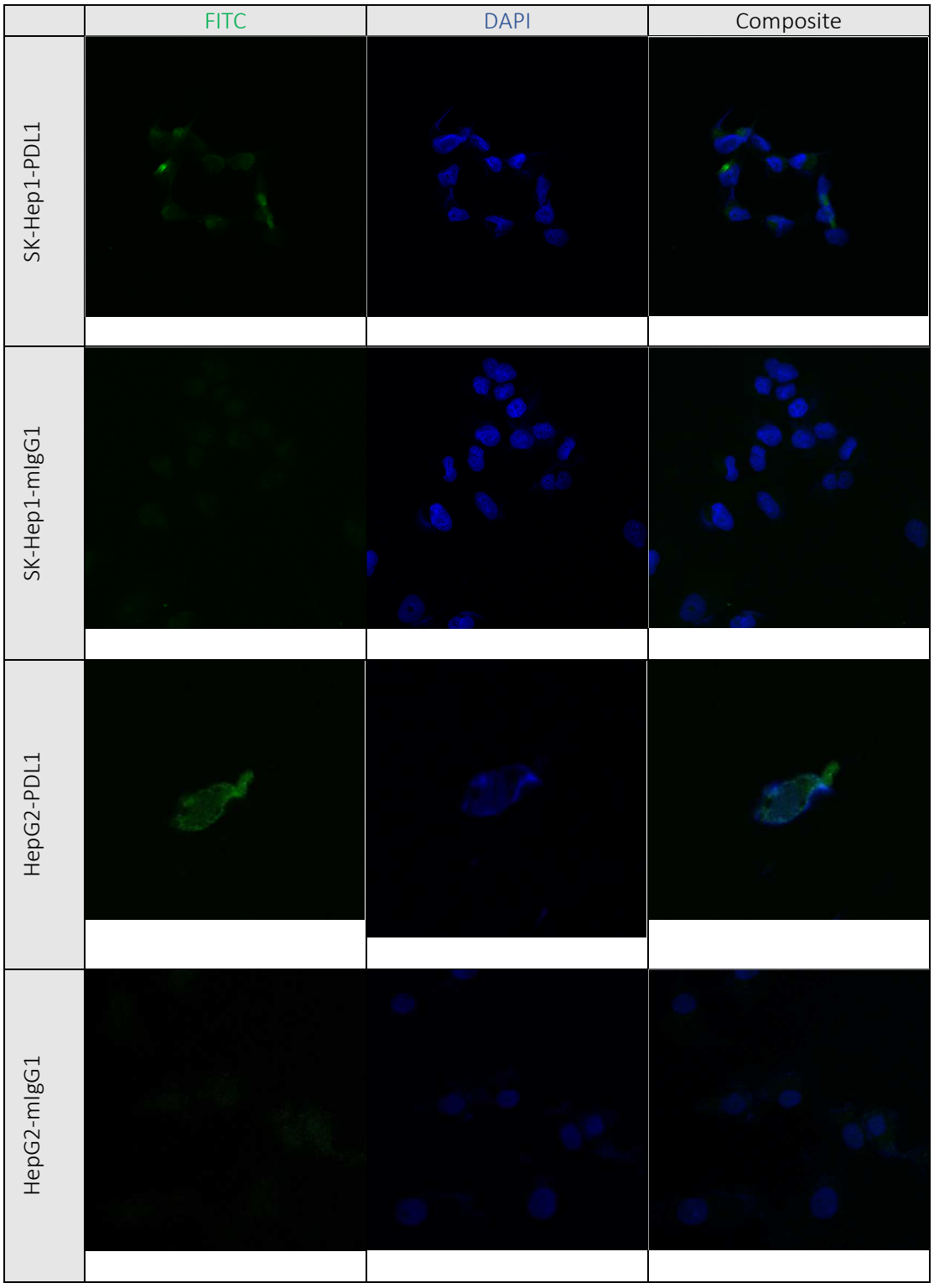
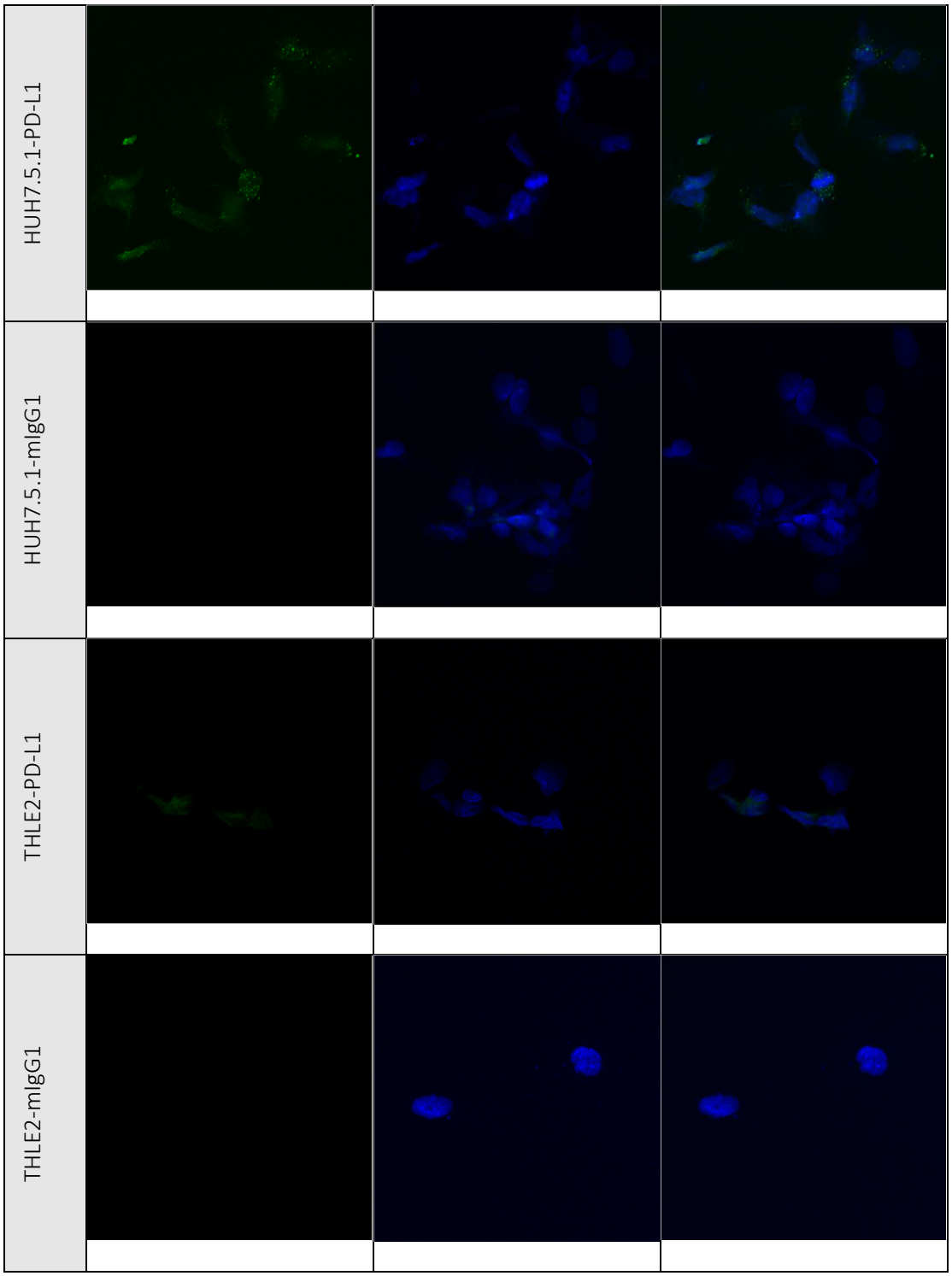

HepG2-LLT1
HepG2-mIgG1

Figure 3.8: PD-L1 is expressed at the cell surface in HCC cell lines. An isotype control (mIgG1 followed by secondary staining with FITC) was used for each of the cell lines to normalize FITC intensity. Split channels/composites were generated using ImageJ software. FITC staining represents expression of PD-L1. Counterstaining with DAPI was done to show expression of PD-L1 relative to the nucleus.


FITC
DAPI
Composite
SK-Hep1-PDL1
SK-Hep1-mIgG1
HepG2-PDL1
HepG2-mIgG1


HUH7.5.1-PD-L1
HUH7.5.1-mIgG1
THLE2-PD-L1
THLE2-mIgG1

Specific Aim 2 - Determine NK cell cytolytic activity against HCC cells when using combination therapy of anti-LLT1 and anti-PD-L1 monoclonal antibodies.

Rationale for Specific Aim 2

Blockage of the LLT1 and PD-L1 ligands using anti-LLT1 and anti-PD-L1 monoclonal antibodies have been shown to increase NK cell activity against cancer cells. With treatment options exploring combination therapies, especially for complicated cancers such as HCC, upregulation of NK cell activity via multiple different treatments could prove beneficial for overcoming the complicated tumor microenvironment seen. Recently, research has suggested the potential of combining anti-PD-L1 monoclonal antibody treatment with NK cell immunotherapy to not only enhance the NK cell response, but activate CD8+ T cell populations as well[70]. Synergistic efficacy of combined immunotherapy has been demonstrated by combining antibodies against CTLA-4 and PD-1, but additional targets need to be explored. LLT1 could provide an excellent second target with PD-L1 because both have been implicated in cancer severity and downregulation of immune cell activity. The purpose of this aim is to explore the efficacy and potential synergistic benefits of combining anti-LLT1 monoclonal antibody treatment with anti-PD-L1 treatment.

3.2 A combination immunotherapy of anti-LLT1 mAb + anti-PD-L1 mAb upregulates NK cell activity more significantly compared to monotherapy treatment.

3.2.1 *Effectiveness of using Combination Immunotherapy of anti-LLT1 mAb + anti-PD-L1 mAb*

Cytotoxicity assays utilizing primary NK cells as effector cells were conducted against HCC cell lines. Samples were run in doublets or triplets and averaged. Percent lysis of THLE2 cells at Effector to Target (E:T) ratios of 25:1, 5:1 and 1:1 for the mIgG1 treated group were 25%, 6%, and 28%, respectively. For the LLT1 treated group, lysis was 0%, 23%, and 22%. For the PD-L1 treated group, percent lysis was 17%, 0% and 27%, respectively. The combination treatment using anti-LLT1+anti-PD-L1 monoclonal antibodies demonstrated 28% lysis, 15% lysis and 6% lysis. Collectively, this data demonstrates that monoclonal antibody treatment with anti-LLT1 mAb, anti-PDL1 mAb, or combined anti-LLT1 + anti-PDL1 mAb did not differ significantly from the negative control. Hopefully, this would be translatable into a host where minimal off-target effects would occur when utilizing this form of treatment. For SK-Hep1 cells, the same E:T ratios were observed (25:1, 5:1, and 1:1). The mIgG1 treated group showed 24% lysis, 19% lysis, and 11% lysis, respectively. The LLT1 treated group showed 35% lysis, 39% lysis, and 28% lysis. The PD-L1 treated group showed 59% lysis, 53% lysis and 39% lysis and the combination treated group of LLT1+PD-L1 showed 52% lysis, 43% lysis, and 43% lysis. Cytotoxicity assays were also performed using the HepG2 cell line at the same E:T ratios. The mIgG1 treated group showed 11% lysis, 10% lysis, and 21% lysis, respectively. The LLT1 treated group showed 18%, 13%, and 13%, respectively. PD-L1 treated group showed percent lysis of 8%, 0%, and 0%, respectively. Lastly, the combination treated group with anti-LLT1+anti-PD-L1 monoclonal antibodies showed 85% lysis, 59% lysis and 63% lysis. Hep3B cells treated with mIgG1 at the same ratios of 25:1, 5:1, and 1:1 showed 16% lysis, 42% lysis, and 6% lysis, respectively. LLT1 treated showed 63%

lysis, 21% lysis, and 17% lysis, respectively. PD-L1 treated Hep3B cells showed 35% lysis, 20% lysis, and 49% lysis, respectively. The combination treated Hep3B cells showed 52% lysis, 70% lysis, and 69% lysis, respectively. HUH7.5.1 cells were also used in a cytotoxicity assay and lysis of the mIgG1 group at 25:1, 5:1 and 1:1 ratios were as follows: 23%, 14%, and 32%. For the LLT1 treated group, lysis percentages were 21%, 31%, and 31%. For the PD-L1 treated group, percent lysis was 18%, 14%, and 21%. For the LLT1+PD-L1 treated group, percent lysis was 89%, 74%, and 76%.

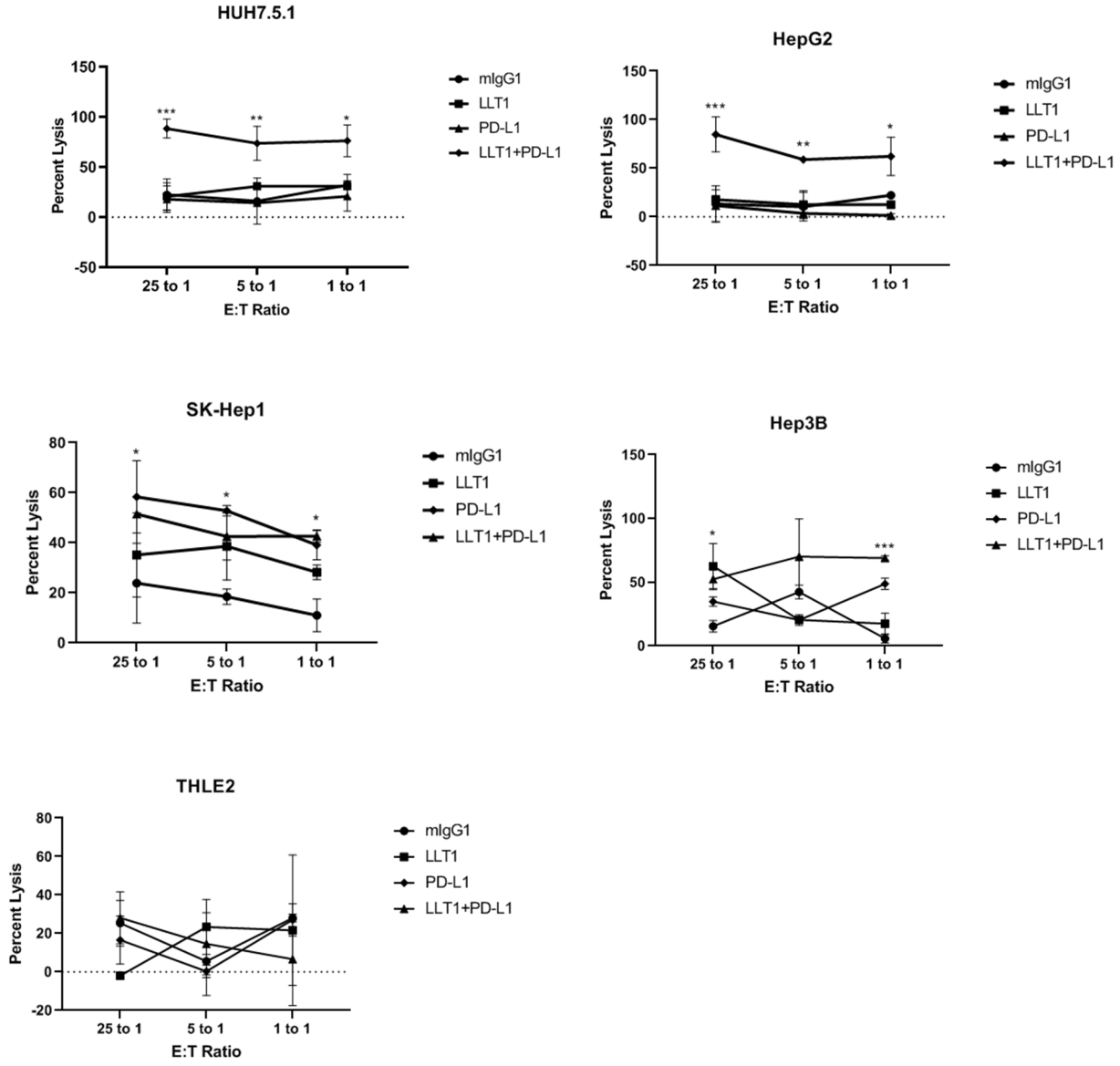
Percent lysis of the isotype control (mIgG1) was compared to each of the monotherapies (anti-LLT1 mAb, anti-PD-L1 mAb) and anti-LLT1 mAb + anti-PD-L1 mAb for each cell line. Non-tumorigenic THLE2 cells did not show significance between any of the treatment groups. SK-Hep1 cells showed significance between mIgG1 and PD-L1 at all E:T ratios (p-values 0.010, 0.010, 0.03) and between mIgG1 vs. LLT1+PD-L1 at E:T ratios of 25:1 and 1:1 (p-values 0.036, 0.017, respectively). HepG2 cells showed significance between mIgG1 and LLT1+PD-L1 at all E:T ratios (p-value 0.0002, 0.004, 0.017). HUH7.5.1 showed significance between mIgG1 vs. LLT1+PD-L1 at all E:T ratios (p-values 0.0009, 0.003, 0.016). Hep3B showed significance between mIgG1 vs. LLT1 at an E:T ratio of 25:1 (p-value 0.003), mIgG1 vs. LLT1+PD-L1 at an E:T ratio of 25:1 (p-value 0.014), mIgG1 vs. PD-L1 at an E:T ratio of 1:1 (p-value 0.005), and mIgG1 vs. LLT1+PD-L1 at an E:T of 1:1 (p-value 0.0003).

To show the specific percent lysis of the cell lines when using the combination treatment and compare this percent lysis between cell lines, the isotype control treatment group was subtracted from the combination therapy. The resultant percent lysis was considered the specific lysis induced by the combination therapy and statistical analysis was performed to compare the non-tumorigenic hepatocyte cell line to the HCC cell lines. At an E:T ratio of 25:1, significance

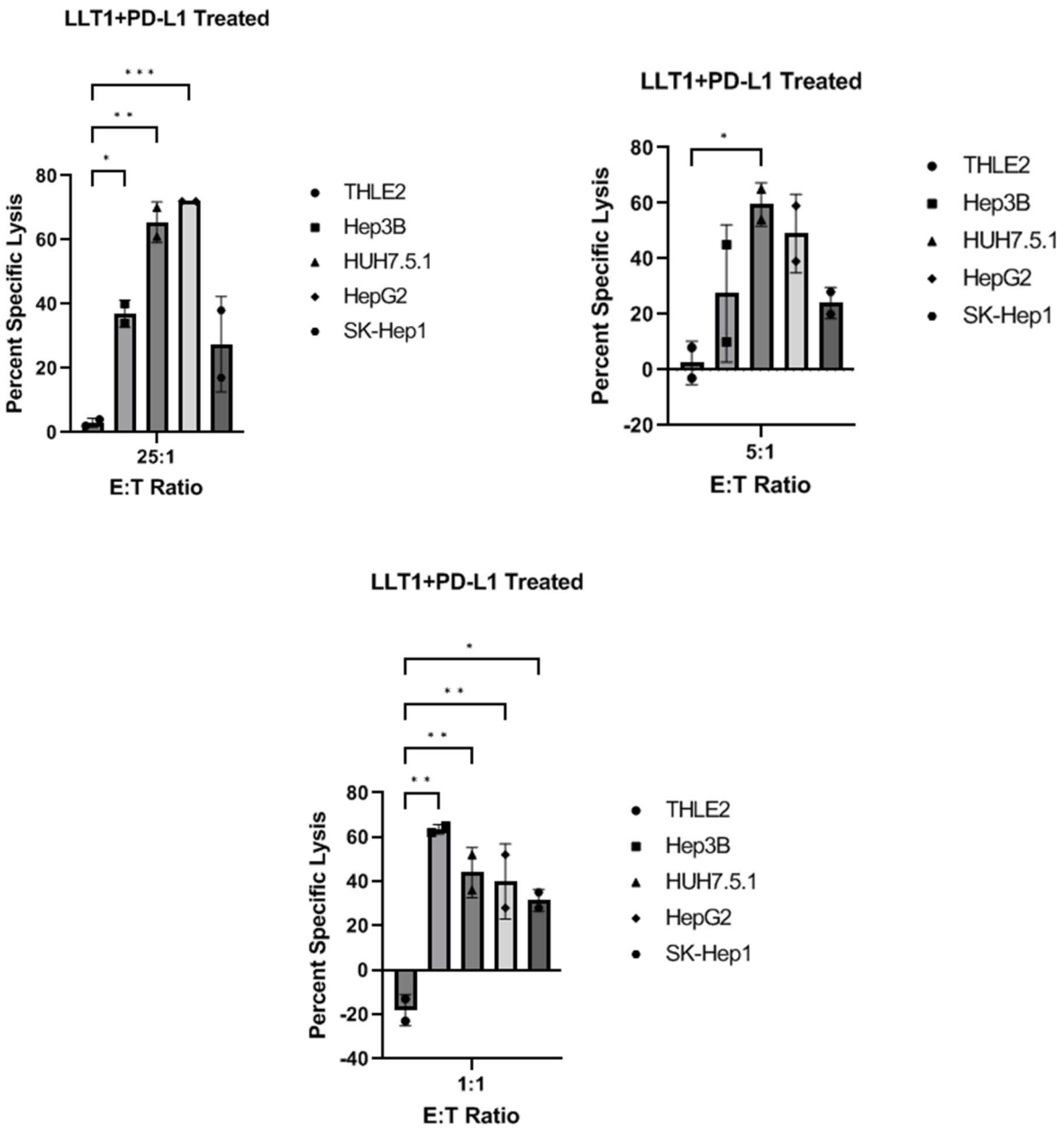
was noted between THLE2 vs. Hep3B (p-value 0.018), THLE2 vs. HUH7.5.1 (p-value 0.001), THLE2 vs. HepG2 (p-value 0.0008). Significance was not noted between and THLE2 and SK-Hep1 (p-value 0.06). At an E:T ratio of 5:1, significance was only noted between THLE2 vs. HUH7.5.1 cells with a p-value of 0.027. At an E:T ratio of 1:1, significance was noted between THLE2 vs. Hep3B (p-value 0.0013), THLE2 vs. HUH7.5.1 (p-value .004), THLE2 vs. HepG2 (p-value 0.006), and THLE2 vs. SK-Hep1 (p-value 0.012).

Figure 3.9: **Anti-LLT1+anti-PD-L1 monoclonal antibody treatment enhances lysis of HCC cells compared to monotherapy.** Cytotoxicity assays showing percent lysis of cancer cells based on treatment with mIgG1 (negative control), anti-LLT1 monoclonal antibodies, anti-PD-L1 monoclonal antibodies or anti-LLT1+anti-PD-L1 monoclonal antibodies. Experiments were run in doublets and averaged, using max lysis and background wells for positive and negative controls, respectively. Statistical analysis to compare treatment groups included a one-way ANOVA followed by Dunnett's multiple comparisons test. P-value<0.05 is represented by *, p-value<0.001**, p-value<0.0001***. A) Percent lysis of cell lines based on treatment. B) Percent specific lysis of cell lines treated with the combination therapy of anti-LLT1+anti-PD-L1 mAbs.

A)


HUH7.5.1
Percent Lysis
E:T Ratio
25 to 1
5 to 1
1 to 1
mIgG1
LLT1
PD-L1
LLT1+PD-L1
HepG2
SK-Hep1
Hep3B
THLE2

B)


LLT1+PD-L1 Treated
Percent Specific Lysis
80
60
40
20
0
25:1
E:T Ratio
THLE2
Hep3B
HUH7.5.1
HepG2
SK-Hep1
LLT1+PD-L1 Treated
Percent Specific Lysis
80
60
40
20
0
-20
5:1
E:T Ratio
THLE2
Hep3B
HUH7.5.1
HepG2
SK-Hep1
LLT1+PD-L1 Treated
Percent Specific Lysis
80
60
40
20
0
-20
-40
1:1
E:T Ratio
THLE2
Hep3B
HUH7.5.1
HepG2
SK-Hep1

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

4.1 Discussion

Recently, NK cell immunotherapies have been gaining traction as treatments for both hematological and solid tumors. Research investigating potential therapeutic targets remains a critical area as HCC is not very responsive to currently used immunotherapies. Combination immunotherapies focused on upregulating both T and NK cell activity are showing promise for treatment against resistant forms of cancer[71]. LLT1 and PD-L1 are NK-associated markers that have been shown to suppress cytotoxic cell function when expressed on cancer cells, resulting in impaired immune responses. While expression of PD-L1 on the surfaces of cancer cells has been well-established, protein expression of LLT1 in hepatocellular carcinoma cells has not been studied, providing a novel avenue to explore. As virally infected hepatocyte cells have been shown to express LLT1 at the surface and the majority of HCC patients are chronically infected with Hepatitis B or Hepatitis C virus, therapeutics targeting LLT1 could provide multi-faceted benefits[72].

As HCC is characterized by a significantly suppressed microenvironment evident of suppressed NK cell activity, gene data was obtained to determine upregulated expression of certain inhibitory biomarkers. Using the publicly available TCGA database, gene expression of LLT1 (CLEC2D) showed significant upregulation in cancerous tissues compared to noncancerous counterparts as indicated in figure 1a. In addition, this expression was shown to increase with

increasing tumor grade (figure 1b), indicating LLT1 as a potential marker of tumor aggressiveness. This finding was not surprising as advanced HCC tumors often exhibit resistance to a multitude of treatments, a characteristic partially due to prior liver disease and viral infection[73]. In figure 1c, gene expression of LLT1 in Caucasian American, African American, and Asian American populations was compared. It was shown that expression of LLT1 was significantly higher in the Asian American cohort compared to the Caucasian American population. This finding correlates with the disproportionate rate at which Asian Americans acquire viral infection and suggests the potential of higher expression of LLT1 due to co-presentation of viral infection and HCC[74]. Figures 2.1 and 2.2 illustrated the variation in severity depending on expression of LLT1 in early vs. late HCC tumor grade. Cohorts were separated into two groups; early tumor grade (1+2) and late tumor grade (3+4) and divided based on low/medium and high expression of LLT1. In the 1+2 tumor grade group (figure 2.1), expression of LLT1 was not shown to be correlated with percent survival as indicated by the p-value of 0.35. However, in the 3+4 tumor grade group (figure 2.2), high expression of LLT1 was implicated as a marker of aggressiveness as individuals within this category had significantly lower rates of survival compared to the low/medium expression group. This finding emphasizes the need for late-stage therapies that are capable of overcoming the tumor resistance implicated. Reverse transcriptase qPCR was performed next to determine gene expression of LLT1 in HCC vs. non-tumorigenic cell lines and the same trend was observed with HCC cells demonstrating elevated mRNA expression of LLT1 comparatively (figure 4.3). Although surface expression of PD-L1 has been well-established in HCC cells, confirmation experiments were performed alongside LLT1 characterization experiments to quantify the expression in the designated cell lines. qPCR analysis of PD-L1 interestingly showed downregulation compared to the non-tumorigenic cell line (figure 4.3). Since gene expression is

not always directly translatable to protein expression where it is functionally relevant, a western blot was done to determine total protein expression of both LLT1 and PD-L1 in HCC and non-tumorigenic hepatocyte cell lines (figure 4.4). Consistent with the qPCR data obtained, protein expression of PD-L1 in the HCC cell lines was lower than in the non-tumorigenic hepatocytes with the exception of the SK-Hep1 cell line, which showed high expression of PD-L1. Although this contradicts the qPCR data demonstrating lower levels of PD-L1 in SK-Hep1, gene and protein expression do not always directly correlate.

As LLT1 and PD-L1 have been shown as expressed both intracellularly and on the surface of certain cells, flow cytometry was performed to determine protein expression at the surface level where interactions with immune cells could be occurring in an inhibitory fashion (figure 4.5). Ultimately, this experiment determined the potential of the ligands as immunotherapeutic targets. Results showed that surface expression of LLT1 was significantly upregulated in HCC cell lines compared to non-tumorigenic counterparts, implicating this marker as a potential mediator of immunosuppressive effects. Additionally, surface expression of PD-L1 was also elevated compared to the non-tumorigenic hepatocyte cell line, but this upregulated expression was not as drastic as LLT1 and did not show statistical significance.

Once surface expression of both LLT1 and PD-L1 was confirmed and this expression was shown as lower in the non-tumorigenic cells, functional assays were performed to explore the functional relevance of these ligands. Previous studies have demonstrated NK cell suppressive effects of both LLT1 and PD-L1, but research has not been performed on LLT1 in HCC[75, 76]. Cytotoxicity assays were conducted using the indicated cell lines (target cells) and primary NK cells (effector cells) isolated from the peripheral blood (figure 4.8). In HepG2 cells, results showed that treatment with an anti-LLT1 mAb combined with an anti-PD-L1 mAb had enhanced lysis

compared to the isotype control and anti-LLT1 or anti-PD-L1 individually. Interestingly, anti-LLT1 mAb and anti-PD-L1 mAb monotherapies induced lytic capabilities similar to the control, indicating minimal efficacy. Numerically, the anti-LLT1 mAb treated group had increased lysis compared to the isotype, however, the anti-PD-L1 group demonstrated decreased lysis compared to the isotype. This could be at least partially due to the relatively low expression of both ligands expressed on this cell line in particular but does not account for the seemingly synergistic effect of combining the two therapies. Effectiveness could be related to intrinsic factors such as HepG2 cells possessing minimally resistant characteristics when targeted from multiple angles with NK cell immunotherapy, or LLT1 and PD-L1 could simply be two major mediators of effector cell activity in this cell line. HUH7.5.1 cells demonstrated a similar trend to the HepG2 cell line with the combination therapy showing significantly enhanced lysis of HCC cells compared to the monotherapies. Again, a synergistic effect appears to be occurring due to the combination treatment. Hep3B cells also had enhanced lysis in the combination treatment group. In the SK-Hep1 cell line, anti-PD-L1 mAb monotherapy was the most successful at inducing NK cell lysis, followed by the combination therapy of anti-LLT1+anti-PD-L1 mAb and finally anti-LLT1 mAb monotherapy. While surprising that the combination group did not have the highest lysis, these results were still promising as it indicates the immunotherapeutic potential of all three forms of treatment to upregulate NK cell activity. Lastly, the THLE2 cell line did not show significant variation in lysis across the three groups included (anti-mIgG1, anti-LLT1, anti-LLT1+PD-L1) indicating that the combination therapy may not cause significant lysis of off-target tissues.

Percent specific lysis of HCC cells compared to non-tumorigenic hepatocytes at all E:T ratios with combination treatment was shown to compare the percent lysis of HCC cells vs. non-tumorigenic hepatocytes. Although somewhat difficult to compare due to variation in cell line

characteristics, these figures demonstrate a clear trend in HCC cell line susceptibility to the combination therapy compared to the non-tumorigenic hepatocytes. This relationship illustrates the potential of using combination immunotherapy blockades against LLT1 and PD-L1 without significant off-target damage.

Although enhanced lysis of HCC cells with the combination therapy was expected, there was minimal success of the monotherapies in upregulating NK cell activity against HCC cell lines, even though high surface expression was detected. These results could indicate that LLT1 and PD-L1 are expressed on different HCC cells, resulting in maximal lysis when combination treatment is employed. Alternatively, since HCC has been indicated as particularly resistant to treatment, there could be multiple receptor-ligand interactions suppressing activity. As such, use of two different mAbs to block inhibitory interactions and induce ADCC could be working synergistically to overcome the suppressive microenvironment, allowing NK-mediated lysis of the HCC cells. This synergy has been demonstrated in Melanoma, with research exploring the efficacy of combining ICIs for enhanced cancer cell lysis[77]. Additionally, NK cells were isolated from a healthy donor and expanding cytokines (IL-2 or IL-15) were not used, so multiple inhibitory blockades could be necessary to effectively activate the NK cells against the HCC cells.

Collectively, this data illustrates the potential promise of combining anti-LLT1+anti-PD-L1 mAbs for resistant HCC tumors, resulting in enhanced targeting of these cancerous cells. Although there is high variation in demographic information and tumor characteristics between the HCC cell lines used, high percent lysis of the HCC cells was demonstrated in each cell line, illustrating the potential broad usage of this combination immunotherapy. As many currently used therapies for HCC remain minimally successful, especially in later stages of cancer development, identification of additional markers involved in suppression and resistance are needed to

effectively target the cancer. NK cell populations are naturally high in the liver, making up ~40-50% of resident lymphocytes. This high population size emphasizes the importance of normal function for continued homeostatic function and early recognition and lysis of virally infected or cancerous cells. During chronic liver inflammation or viral infection, NK cell subsets are initially activated, then rapidly become exhausted, leaving the liver prone to progression to cancer. Once the liver has progressed to a cancerous state, this immunosuppression of NK cells is further exemplified through cancer cell upregulation of markers involved in inhibition of activity. LLT1 presents as a potential therapeutic target as results indicate the role it plays in suppressing NK-cell activity, in the context of TNBC, Ewing Sarcoma, prostate cancer, HCC and virally infected cells[78, 79]. As HCC patients present with multiple comorbidities often including HCV or HBV, targeting this ligand could enhance not only killing of cancer cells, but also virally infected hepatocytes which express LLT1, working in combination to subdue both the cancer and virus. In addition, activation of both NK cells and CD8+ T cells via PD-L1-PD-1 blockade could work synergistically with LLT1 to overcome the multi-faceted tumor microenvironment.

4.2 Future Investigations

While these results demonstrate the potential of a combination immunotherapy for HCC, further research is needed to explore these effects in primary human tissues. Future experiments include performing a subcellular fractionation would show location-dependent expression of LLT1 in HCC cell lines. Further analysis in primary tissues would be the next step to confirm trends in protein expression, followed by a mouse model to determine the effects in a living host. Ideally, the experiments would be performed in an animal with induced mid to late-stage HCC, viral infection in the form of HBV or HCV, and cirrhosis to appropriately replicate the functional

relevance of anti-LLT1+anti-PD-L1 against HCC. As the HCC microenvironment remains a major obstacle to overcome and decreases in the number of NK cells have been noted in the liver during cancer, additional *in-vivo* research should investigate the efficacy of NK cell transfusions coupled with ICI treatment. Research has indicated that NK cell transfusions independently have resulted in progression-free survival of an HCC patient with significantly increased quality of life, but the NK cells were not able to mount a significant response until ~10 months of transfusions[80]. Coupling transfusions with ICIs or ADCC inducing forms of therapy could potentially accelerate this process of targeted cancer lysis, decreasing progression of the cancer. Ultimately, combination immunotherapies that upregulate NK cell activity could be the future of treatment for HCC as research continues to indicate the crucial anti-cancer and anti-viral role that they play in this organ system.

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