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Hepatocellular carcinoma (HCC) is a global leading cause of cancer-related deaths. Current treatments for HCC are ineffective. Transplantation and surgical resection are the only curative options, however, only an approximate 3% of HCC patients are eligible. Without transplantation or resection, the 5-year, overall survival (OS) rates remain <10%, with death 6-18 months from diagnosis. Cancer immunotherapy provides an avenue to investigate potential treatments as immunotherapies can promote immune-mediated tumor lysis, while sparing patients from some of the toxic effects of radiation and chemotherapy. Prior research suggests the inhibitory mechanisms underlying the LLT1/NKR-P1A interaction attributes to cancer growth as it allows the cancer to evade immune surveillance. Studies demonstrate improved NK cell activity and NK cell-mediated tumor lysis upon applying LLT1 blockades in prostate, breast, and hematological cancers. The goal of this project was to similarly evaluate the role of LLT1 in HCC.

THE FUNCTIONAL ROLE OF LECTIN-LIKE TRANSCRIPT-1 (LLT1) IN HEPATOCELLULAR CARCINOMA

INTERNSHIP PRACTICUM REPORT

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CHAPTER I

INTRODUCTION

Hepatocellular carcinoma (HCC) is a globally leading cause of cancer-related deaths. According to the American Cancer Society, in 2023 an estimated 41,210 Americans will be diagnosed with liver cancer and approximately 30,000 will die. HCC is the most common form of liver cancer and is typically detected at later, more advanced stages. The typical life expectancy from diagnosis is 6-20 months, with only a 10% 5-year survival rate (Golabi *et al.*, 2017). Despite general advances in cancer prevention awareness, detection, and treatment, mortality rates remain high. Additionally, incidence rates are expected to increase with rising rates of alcohol consumption and metabolic-related risk factors including obesity, type-2 diabetes, and non-alcoholic fatty liver disease. Immunotherapy offers an avenue into cancer treatment that can reduce the reliance on toxic treatments, and potentially train the immune system to prevent recurrence (Cohen, 2020).

The purpose of this project was to assess the role of lectin-like transcript 1 (LLT1) and explore the functional outcome of using a combination of LLT1 and programed death-ligand 1 (PD-L1)(CD274) monoclonal antibodies (mAb) in hepatocellular carcinoma. When LLT1 and PD-L1 interact with their receptors, NKR-P1A (CD161) and PD-1 (CD279) respectively, inhibitory signals are transmitted to natural killer (NK) cells. This inhibitory mechanism is critical for immune self-recognition. Studies have shown that particular cancers overexpress these proteins, exploiting this mechanism to evade anti-tumor immune responses (Mathew *et al.*, 2016; Braud *et al.*, 2022).

Previous experiments performed by a former graduate student in Dr. Stephen Mathew's lab showed increased expression of LLT1 and PD-L1 on the surface of HCC cells. To evaluate the function of these cell surface proteins in HCC, the LLT1/NKR-P1A and PD-L1/PD-1 interactions were blocked using mAbs and NK-mediated cytotoxicity was evaluated with CD107a degranulation assays.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

Hepatocellular Carcinoma (HCC)

Currently, HBV and HCV remain the most significant risk factors for developing HCC. However, with increased screening and HBV vaccinations, the role of viral hepatitis in the pathogenesis of HCC is expected to decline. Excess alcohol consumption (5 times more prevalent than HBV in the US) combined with metabolic factors such as obesity, type 2 diabetes, and NAFLD are expected to become the leading risk factors (McGlynn *et al.*, 2020). During inflammation, hepatocyte stellate cells (HSCs) initiate extracellular matrix (ECM) production in the space of Disse (perisinusoidal space) resulting in fibrogenesis. Under normal physiological conditions, this process is reversible upon tissue recovery. However, under a state of chronic inflammation, typically quiescent HSCs transform into myofibroblasts, a type of cancer-associated fibroblast (CAF) (Sas *et al.,* 2022). These CAFs, along with mesenchymal stem cells/stromal cells (MSCs) contribute to the tumor microenvironment (TME) via secreting extracellular matrix (i.e. collagen, proteoglycans, hyaluronan), growth factors (such as EGF and PDGF), chemokines, cytokines, and metalloproteinase (MPP) enzymes. The TME also consists of immune cells, including tumor-associated macrophages (TAMs), natural killer cells, T lymphocytes, and neutrophils (Sevic *et al.,* 2019). The TME results in an immunosuppressed, chronically inflamed environment facilitating a diminished anti-tumor immune response, promotes tumorigenesis, and plays a major role in metastasis.

Treatments

Orthotopic liver transplantation (OLT) or surgical resection remain the only curative options for HCC. With transplantation, the 5-year survival rate increases to greater than 75% and less than 15% chance of recurrence, however, only approximately 5% of patients are eligible to receive a transplant or

resection (Cicalese, 2022). Therefore, it is crucial to develop new therapies which can help bridge more patients to being eligible for surgical, curative options.

Next line of treatment includes radiofrequency ablation, cryoablation, in addition to alcohol and microwave ablation. Chemotherapeutics and radiation can be delivered directly to cancer cells via chemoembolization and radioembolization. Additionally, typical radiation and chemotherapy regimes are approved, such as FOLFOX4 (infusional fluorouracil, leucovorin, and oxaliplatin) (Tian *et al.*, 2018).

Currently, several other therapies are approved for use in hepatocellular carcinoma including sorafenib (a multikinase inhibitor), lenvatinib (a tyrosine kinase inhibitor), and several newer drugs including atezolizumab (a PD-L1 immune checkpoint inhibitor) and bevacizumab (an antiangiogenic agent) (Luo *et al.*, 2022).

Natural Killer (NK) Cells

Derived from hematopoietic stem cells, NK cells play an important role in the innate immune system. They are crucial for the immunosurveillance of virally infected and cancerous cells, inducing apoptosis via the Fas/FasL signaling pathway (Sas *et al.*, 2020). A balance of inhibitory and activating signals determines the activity of NK cells. NK cells identify and destroy aberrant cells through multiple mechanisms. NK cells can directly target virally-infected or cancerous cells through cytotoxic granule release (perforin, granulysin, granzyme), and $CD16^{+}$ NK cells are capable of recognizing the Fc portion of antibodies, inducing antibody-dependent cellular cytotoxicity (ADCC). Additionally, NK cells act indirectly via the production of cytokines such as IFN-γ, TNF-α, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), and IL-33. These, along with other cytokines, recruit additional immune cells to the target (Allison et al., 2022). Receptors on the surface of NK cells interact with ligands on the surface of immune cells, host tissue, and pathogens. When NK cells come into contact with healthy host tissue, inhibitory receptor-ligand interactions override activating signals to the NK cells to prevent lysis of normal healthy cells and maintain tolerance in the body.. However, when NK cells come into contact with

virally infected or cancerous cells, activating receptor-ligand interactions stimulate cytotoxic granule release and cytokine production, ultimately killing the virally infected or cancerous cell (Bláha et al., 2021). Additionally, evidence suggests that NK cells are capable of recognizing a variety of antigens, akin to a memory-like immune response (Cooper, 2018).

Lectin like-transcript 1 (LLT1)

LLT1 is a protein expressed on the surface of lymphocytes, such as NK cells, T and B cells, as well as activated dendritic cells. LLT1 is a member of the C-type lectin-like receptor superfamily (CTLR) and is encoded by the CLEC2D gene located in the human natural killer gene complex (Marrufo et al., 2018). The ligand for LLT1 is NKR-P1A (CD161), which is expressed on the surface of all NK cells (in addition to other immune cells). NKR-P1A is encoded by the KLRB1 gene. The LLT1/NKR-P1A interaction inhibits NK cell activity. Cancerous cells are capable of hijacking this inhibitory mechanism to evade immune anti-tumor responses.

The discovery of LLT1 overexpression in numerous cancers, and its role in immune surveillance evasion, has led researchers to study the functional role of this protein (Buller et al., 2020). Studies have shown improved NK cell-mediated lysis of tumor cells when blocking the LLT1/NKR-P1A interaction in a variety of cancers, including triple negative breast cancer (Marrufo et al., 2018) and prostate cancer (Mathew et al., 2016). Additionally, evidence suggests that LLT1 may be used as a potential prognostic factor (Sanchez-Canteli et al., 2020).

Programmed death-ligand 1 (PD-L1)

Programmed death-ligand 1 (PD-L1) (CD274) is expressed on the surface of macrophages, some activated T and B cells, dendritic cells and tumor cells. PD-L1 interacts with its receptor, programmed cell death protein 1 (PD-1) (CD279), expressed on the surface of a variety of immune cells including NK cells, T and B lymphocytes, monocytes, and macrophages (Wu et al., 2019). Similar to the LLT1/NKR-

P1A interaction, the PD-L1/PD-1 interaction functions as an inhibitory immune checkpoint (IC). This mechanism is critical for immune self-recognition. The use of anti-PD-1 monoclonal antibodies in nude mice resulted in increased NK cell cytotoxic activity and increased tumor-infiltrating PD-1^+ NK cells. This evidence suggests a crucial role for PD-1 on NK cells, particularly in cancers of the digestive tract (Poggi and Zocchi, 2021).

In murine models, the interaction of PD-1 on NK cells with PD-L1-expressing tumor cells reduced NK cell cytotoxicity. However, when the PD-1/PD-L1 interaction was blocked, NK cell activity was restored. Additionally, these restored NK cells were able to produce cytokines and chemokines. (Poggi and Zocchi, 2021). Furthermore, blocking of the PD-1/PD-L1 interaction (in addition to a CTLA4 immune checkpoint block) has also provided good clinical results in specific human cancers, including gastric cancers, lung cancers, and melanomas (Callahan and Wolchok, 2013).

HYPOTHESIS

Hypothesis: Blocking the LLT1/NKR-P1A and PD-L1/PD-1 interactions will increase NK cell-mediated cytotoxicity in HCC. We will test the hypothesis by the following aims:

Aim 1: Evaluate NK cell-mediated lysis of HCC tumor cells when the LLT1/NKR-P1A and PD-L1/PD-1 interactions are blocked using anti-LLT1 and anti-PD-L1 mAbs.

SIGNIFICANCE

With ineffective treatments, a failure to improve progression-free survival (PFS) and/or over survival (OS) rates, and a high burden from treatment side effects, it is important to develop improved

methods of treatment. Immunotherapy is beneficial as it uses the body's natural defense mechanisms leading to fewer adverse effects. Additionally, studies show that NK cells can lower recurrence as they have the potential to develop immunologic memory.

Studies involving other cancers (prostate, TNBC) (Mathew *et al.*, 2016; Marrufo *et al.*, 2018) demonstrate increased NK cell mediated tumor lysis upon blocking LLT1 and PD-L1 with the use of mAbs. As data suggests an overexpression of LLT1 and PD-L1 on the surface of HCC cells provides justification to further explore LLT1 and its functional role in hepatocellular carcinoma.

MATERIALS AND METHODS

Cell Lines and Culture

Human cell lines Hep3B (ATCC HB-8064), SK-Hep1 (ATCC HTB-52), and HUH7.5.1 were used as models of hepatocellular carcinoma. Hep3B was obtained from the liver of an 8-year old, black youth, and is infected with HBV. SK-Hep1 was isolated from a 52-year old white male suffering from hepatic adenocarcinoma. HUH7.5.1 was derived from the liver tumor of a 57-year old Japanese male. Additionally, HUH7.5.1 is highly susceptible to HCV infection. All HCC cell lines were cultured in Eagle's Minimum Essential Medium (Corning Life Sciences, Teterboro, NJ) supplemented with 10% fetal bovine serum (Atlanta Biologicals Inc, Lawrenceville, GA), 1% non-essential amino acids (Cytiva, Marlborough, MA), 1% sodium pyruvate (Cytiva, Marlborough, MA), 1% antibiotic and antimycotic (Thermo Fisher Scientific, Waltham, MA). Additionally, the medium used for Hep3B was supplemented with plasmocin per manufacturer (Invivogen, Toulouse, France). All cell lines possess epithelial morphology, with the exception of SK-Hep1, which has been reclassified as having endothelial morphology.

RNA Isolation & Quantitative PCR (qPCR)

RNA was extracted from cells using TRIzol (Invitrogen, Waltham, MA) and the concentration and purity were determined with a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNA was then converted into cDNA using Maxima H-Minus cDNA Synthesis Master Mix with dsDNase (Thermo Fisher Scientific, Waltham, MA). Additionally, the Nanodrop Lite was used to determine the concentration and purity of the cDNA. PCR was performed on the cDNA product and subsequently ran on 0.5g agarose gels (Thermo Fisher Scientific, Waltham, MA) for confirmation of the expression of LLT1 and PD-L1.. Next, qPCR was performed in triplicate using TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA). To account for background noise, the reference dye ROX is included in the mix. GAPDH primers were used as a reference gene for both PCR and qPCR.

Flow Cytometry

Flow cytometry was performed on a Cytek Aurora flow cytometer to evaluate the cell surface expression of LLT1 and PD-L1. Phycoerythrin(PE)-conjugated mouse anti-human anti-LLT1 antibodies (R & D Systems, Minneapolis, MN) and brilliant violet(BV) 421-conjugated mouse anti-human anti-PD-L1 antibodies (Biolegend, San Diego, CA) were used to assess protein surface expression. PE-conjugated mouse IgG1 and BV421-conjugated IgG2b (Biolegend, San Diego, CA) were used as the respective LLT1 and PD-L1 isotype controls to account for non-specific antibody binding. The samples were analyzed using FlowJo software and mean fluorescent intensity ratios (MFIRs) were determined as a ratio of live cells positive for LLT1/PD-L1 to live cells positive for the corresponding isotype controls. An MFRI greater than 1 is indicative of a positive expression of said protein(s).

Isolation of Primary NK Cells

Whole-blood samples were obtained from a healthy donor with approval (IRB #20-28) from the Internal Review Board of UNT Health Science Center, Fort Worth, TX. Ethylene-diamine-tetraacetic acid (EDTA)-treated blood samples were treated with Lymphoprep (StemCell Technologies, Cambridge, MA) density gradient centrifugation using SepMate tubes (StemCell Technologies, Cambridge, MA), resulting in isolated peripheral blood mononuclear cells (PBMCs). An NK cell isolation kit (StemCell Technologies, Cambridge, MA) was used to isolate NK from the PBMCs. Flow cytometry using anti-human CD56 mAb was used to determine purity.

CD107a Degranulation Assay

Upon treating isolated effector NK cells with anti-human IgG Fc antibodies (Rockland Immunochemicals, Inc. Pottstown, PA), the NK cells were stained with anti-human CD107a (Biolegend, San Diego, CA) and resuspended in RPMI 1640 medium (ATCC, Manassas, VA) containing 10% FBS (Atlanta Biologicals Inc, Lawrenceville, GA). Target HCC cells were harvested and stained with CFSE (Biolegend, San Diego, CA). Experimental groups consisted of untreated, anti-human LLT1 block (Invitrogen, Waltham, MA), anti-human PD-L1 block (R & D Systems, Minneapolis, MN), and combination LLT1 and PD-L1 blocks. Effector and target cells (25:1) were co-incubated in phorbol-12-myristate-13-acetate (PMA) (2.5 μg/mL) and ionomycin (0.5 μg/mL) as a positive control. Negative controls included effector cells only, in addition to a spontaneous death control (target cells only). Appropriately conjugated mouse IgG1 (Biolegend, San Diego, CA) was used for all respective isotype controls. Effector and target cells were co-incubated in 96-well V-bottom plates with effector to target ratios of 25:1, 5:1, and for four hours at 37°C. Upon removing the plate from the incubator, 10 μL of 10 μL/mL propidium iodide (PI) (Thermo FIsher Scientific, Waltham, MA) was added to each well. One-way ANOVA was used to compare NK cell degradation in the presence of HCC cells with and without LLT1 and/or PD-L1 blockades.

RESULTS AND DISCUSSION

To confirm the presence of *Clec2d* (LLT1) and *CD274* (PD-L1) mRNA among different HCC cell lines, qPCR was performed. GAPDH was used as a housekeeping gene. The average ΔCT for each cell line from three experiments was calculated and analyzed in Prism GraphPad. Because a non-tumorigenic cell line was unavailable, HUH 7.5.1 (expressing the lowest mRNA) was normalized to one and used as a control for calculating the fold-change. Expression of *Clec22d* (LLT1) and *CD274* (PD-L1) was confirmed for all three cell lines used. SK-Hep1 showed two-fold higher expression for PD-L1 than HUH 7.5.1 and Hep3B cell lines whereas HUH 7.5.1 showed maximum LLT1 mRNA expression as compared to SK-Hep1 and H3B (Fig. 1).

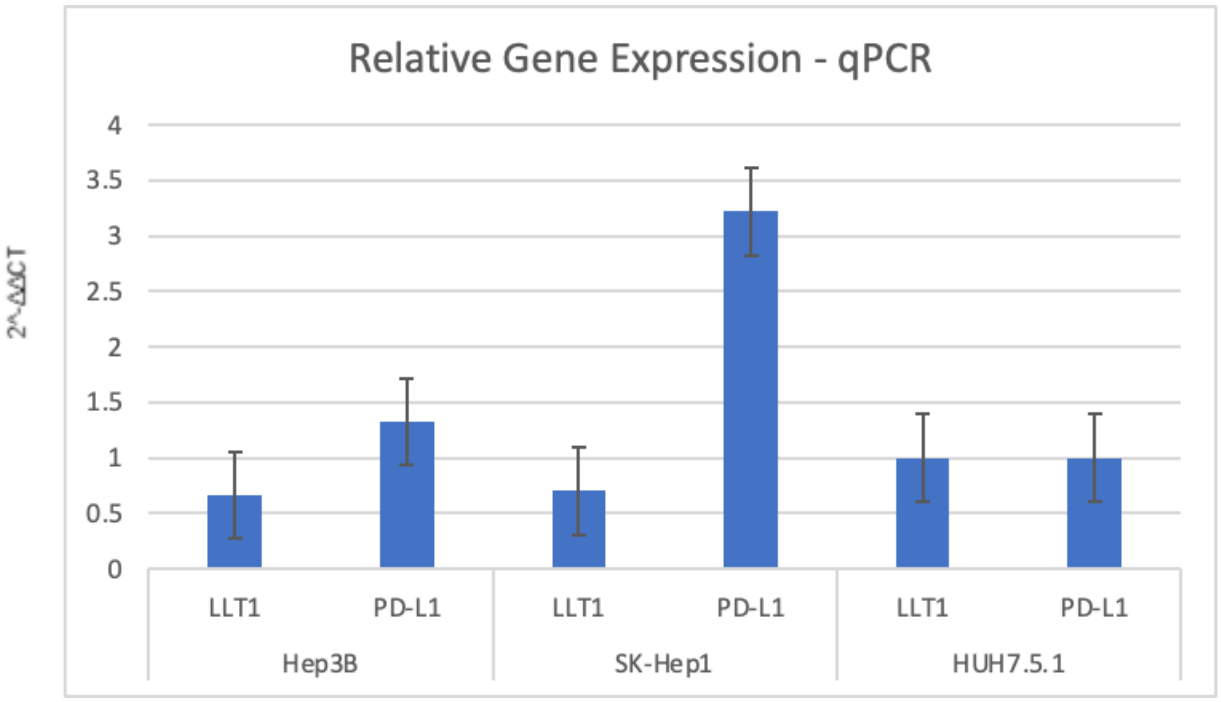

Figure 1. mRNA expression of LLT1 and PD-L1 in HCC cell lines. qPCR using sequence specific primers for LLT1, PD-L1, and GAPDH was performed. Each bar represents the fold change ± SD relative to HUH 7.5.1. Fold change was determined using the average ΔCT of each cell line from three experiments.

To evaluate cell surface expression of LLT1 and PD-L1 in HCC, flow cytometry was performed. Isotype controls (mIgG1) were used as negative controls and represented as a red histogram in the figures. Test groups were created using anti-LLT1 mAbs, anti-PD-L1 mAbs, and a combination of anti-LLT1 and anti-PD-L1 mAbs (yellow histograms). Mean fluorescent intensities (MFIs) were analyzed using FlowJo software and the experimental group/isotype control ratio was calculated (MFIR). An MFIR greater than one is indicative of upregulation in surface expression. A trend in the histograms demonstrates surface expression of LLT1 and PD-L1 (Fig. 2 - 4).

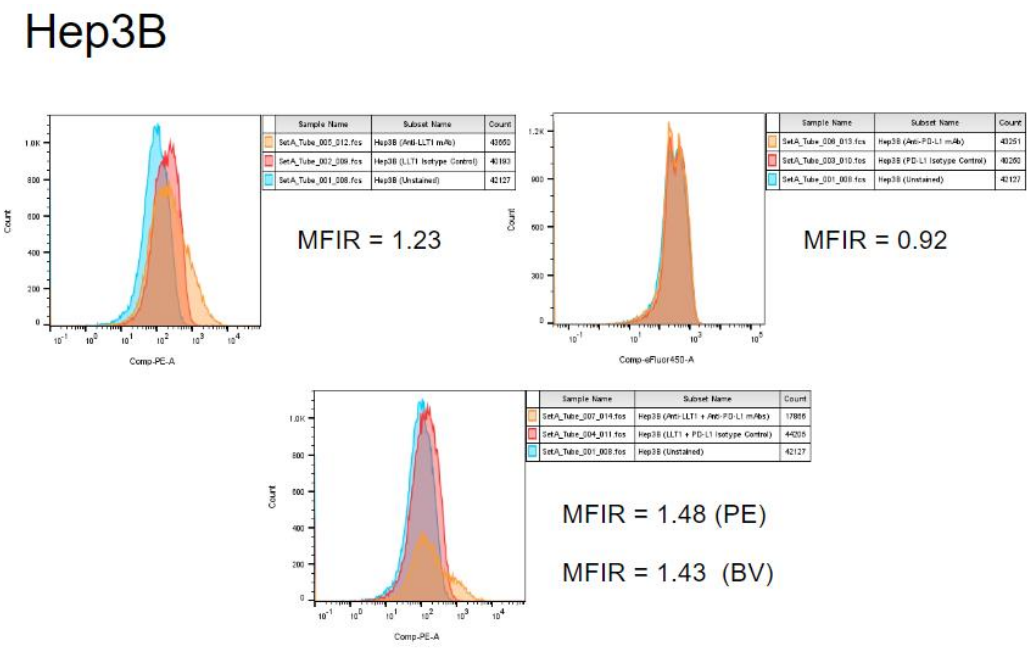

Figure 2. Flow cytometry histograms for LLT1 and PD-L1 expression (Hep3B). The yellow peaks represent the respective anti-human monoclonal antibodies; red peaks indicate mIgG1 isotype control; Blue peaks are unstained/ (Top left) Anti-human LLT1 mAbs; (Top right) Anti-human PD-L1 mAbs; (Bottom) Anti-human LLT1 + PD-L1.

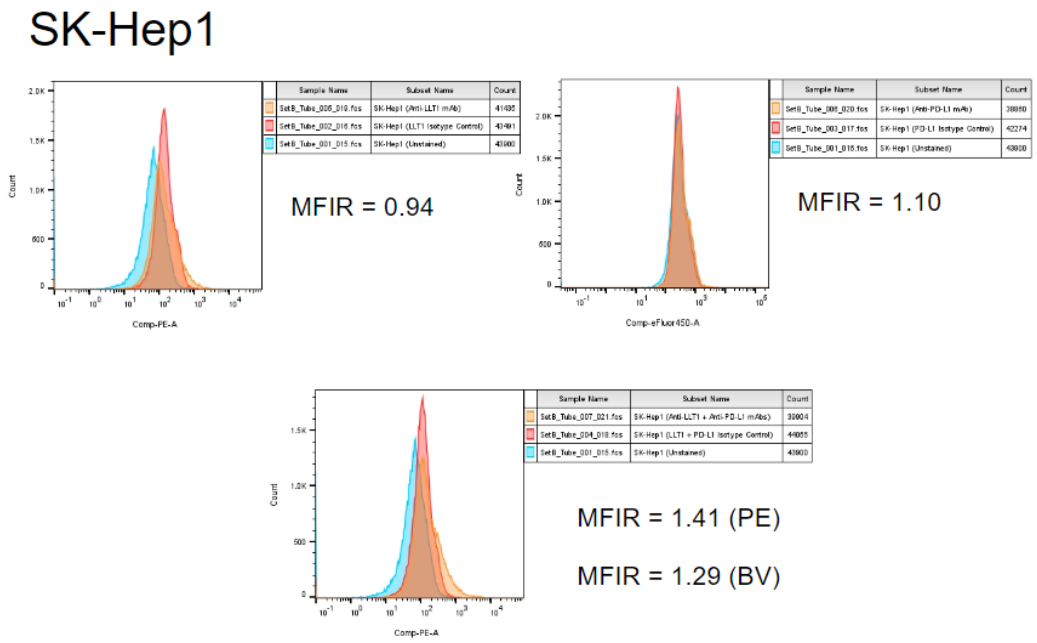

Figure 3. Flow cytometry histograms for LLT1 and PD-L1 expression (SK-Hep1). The yellow peaks represent the respective anti-human monoclonal antibodies; red peaks indicate mIgG1 isotype control; Blue peaks are unstained/ (Top left) Anti-human LLT1 mAbs; (Top right) Anti-human PD-L1 mAbs; (Bottom) Anti-human LLT1 + PD-L1.

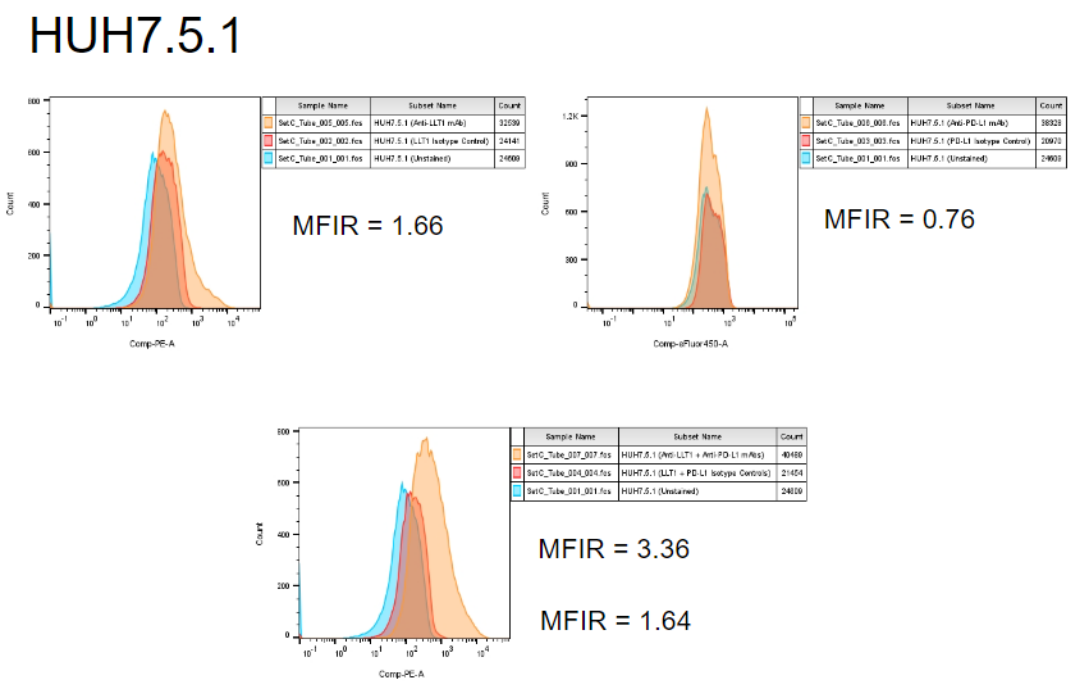

Figure 4. Flow cytometry histograms for LLT1 and PD-L1 expression (HUH 7.5.1). The yellow peaks represent the respective anti-human monoclonal antibodies; red peaks indicate mIgG1 isotype control; Blue peaks are unstained/ (Top left) Anti-human LLT1 mAbs; (Top right) Anti-human PD-L1 mAbs; (Bottom) Anti-human LLT1 + PD-L1.

Natural killer cells were stained with anti-CD107a-PE and anti-CD56-APC mAbs. The cells were then incubated with HCC cells at E:T ratios of 25:1, 5:1, and 1:1 for four hours. A negative isotype control mIgG1 was used. The test was performed in triplicate and a one-way ANOVA (Prism GraphPad) was used to compare the percentage change among groups of $CD56^+$ / $CD107a^+$ cells. CD56 is a marker for NK cells, while CD107a is a functional biomarker for evaluating NK cell activity. A hallmark of NK cell activation is degranulation, that is, the release of lytic granule contents (perforin and granzymes) onto the surface of the target cell. After degranulation, CD107a is exposed on the surface of the cytotoxic lymphocyte, where it might protect the outer membrane from perforin-mediated damage (Fukuda, 1991). The data suggests that LLT1-blocked HCC results in increased NK cell activity, thus supporting the hypothesis that increased surface expression of LLT1 in HCC decreases NK cell-mediated lysis (Fig. 5). For some reason the blockade with PD-L1 did not show increased degranulation. The combination of LLT1 and PD-L1 was comparable with LLT1 blockade at 25:1 E:T ratio.

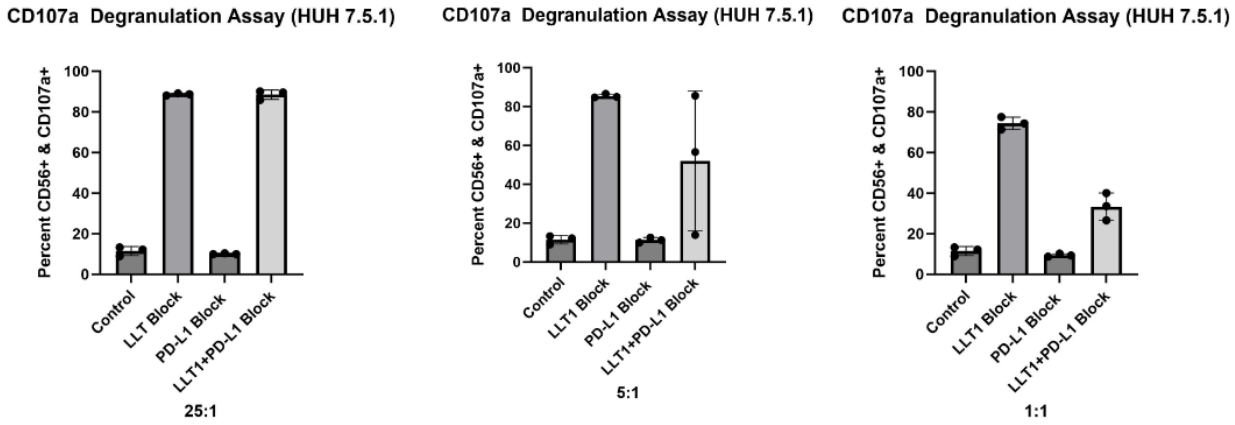

Figure 5. LLT1 is associated with decreased NK cell activity. NK cells were tagged with anti-CD56-APC and anti-CD107a-PE mAbs. NK cells were incubated with anti-human LLT1 and/or PD-L1 mAbs to block the LLT1/NKR-P1A and PD-L1/PD-1 interactions. NK cells and HUH 7.5.1 cells were co-incubated for 4 hours. Flow cytometry was used to isolate $CD56^+$ / $CD107a^+$ NK cells. Bars represent the percentage change of $CD56^+$ / $CD107a^+$ NK cells among experimental groups.

An NK cell cytotoxicity assay was performed in triplicate using primary NK cells as effector cells and HUH 7.5.1 cells as target cells. Prior to co-incubating for 4 hours, HUH 7.5.1 cells were stained with CFSE and the NK cells were treated with an Fc block. LLT1 blocked, PD-L1 blocked, and combination LLT1 + PD-L1 blocked experimental groups were created to compare against a control group (mIgG1). Effector to target ratios of 25:1, 5:1, and 1:1 were used. At the end of incubation, propidium iodide was added. Percent lysis was calculated by subtracting the average of spontaneous death from each of the experimental groups when gating for $CFSE^+$ / PI^+ (dead HCC cells) using FlowJo. One-way ANOVA was performed to determine statistical significance $(P \leq 0.05)$(Prism GraphPad). A clear trend of increased NK cell-mediated HCC lysis in the presence of a LLT1 or PD-L1 or combination blockade of LLT1 and PD-L1 is shown (Fig. 6).

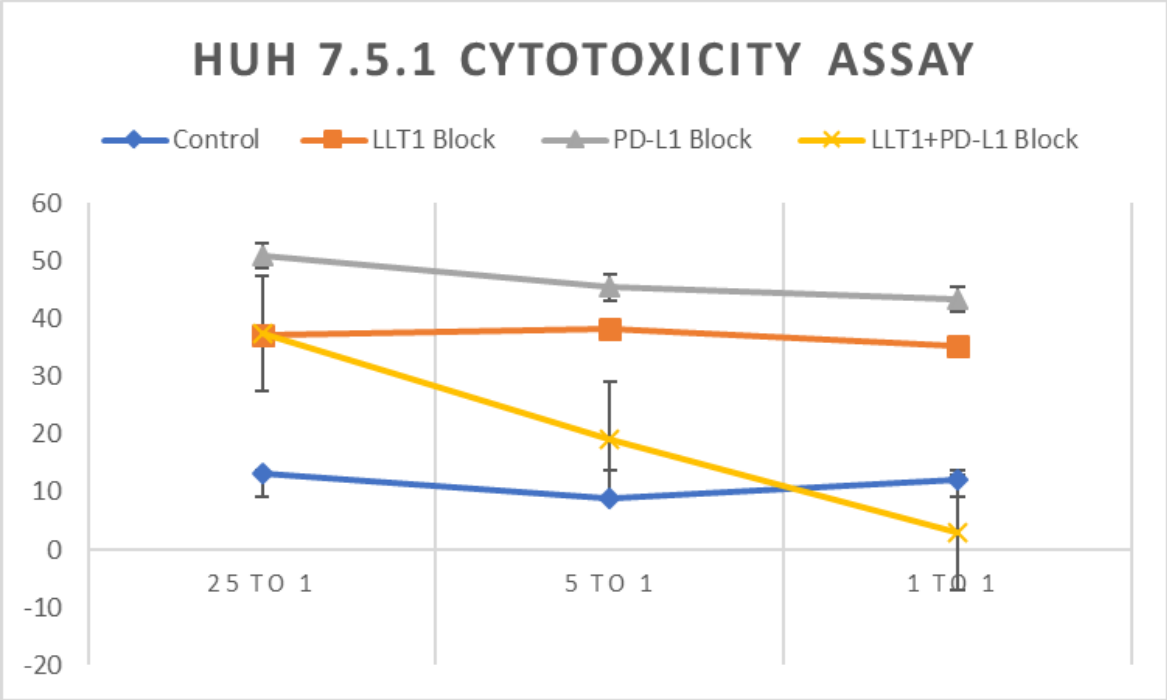

Figure 6. LLT1 blockade results in increased NK cell-mediated HCC tumor lysis. HCC cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and incubated with NK cells for 4 hours in a 96-well plate. Propidium iodide (PI) (10µl/ml) was added and cells were analyzed with flow cytometry. NK-mediated tumor lysis was determined by gating for $CFSE^+$ / PI^+ HCC cells in FlowJo. Average percentage changes between experimental groups were compared to each other and a spontaneous death control using a one-way ANOVA in Prism GraphPad $(P \leq 0.05)$.

SUMMARY AND CONCLUSION

Currently, there are numerous FDA-approved immunotherapies for treating a range of cancers including breast, prostate, lung, and hematological. As the HCC incidence rate continues to increase and mortality rates remain high, alternate treatments are warranted. Immunotherapies provide an additional investigative avenue due to their low side-effect burden, lower toxicity, and potential ability to prevent recurrence. Additionally, evidence suggests that LLT1 is upregulated on the surface of hepatocytes infected with HBV and HCV. As HBV and HCV remain significant risk factors for the development of HCC and a large percent of HCC patients have HBV and/or HCV infections, researching the LLT1/NKR-P1A interaction has the potential to provide multi-faceted benefits (Ringelhan *et al.*, 2017).

An immunosuppressive tumor microenvironment is a major component in HCC pathogenesis. Studies support an immunosuppressive role of the LLT1/NKR-P1A interaction in multiple cancers and upon blocking the interaction there is increased tumor cell lysis by immune cells (Mathew *et al.*, 2021; Braud *et al.*, 2022). qPCR was used to determine the presence and relative mRNA expression of LLT1 in all three cell lines used. As mRNA expression does not necessarily equate surface expression, flow cytometry was also performed to evaluate LLT1 and PD-L1 surface expression. Performing a functional CD107a degranulation and cytotoxicity assays further supports the hypothesis that blocking the LLT1/NKR-P1A interaction (in combination with a PD-L1/PD-1 blockade) increases NK cell-mediated tumor lysis. Therefore, combined LLT1 immunotherapeutics appear to be a valid area for further investigation.

Immortalized cell lines have been the mainstay of pre-clinical biomedical research since their development. They are convenient, cost-effective, and have been used in biomedical research for decades. However, using cell lines *in vitro* poses a limitation as they do not necessarily recreate the *in vivo* environment. Serial passage of immortal cell lines leads to genotypic and phenotypic changes that can lead to findings that are not physiologically relevant (PromoCell, 2019).

A lack of non-tumorigenic hepatocytes is a limitation due to the inability to compare mRNA expression, surface expression, and functional assay results between non-tumorigenic cells and HCC. Additionally, due to time constraints the functional assay was performed on only one HCC cell line.

Future research should confirm mRNA expression, surface expression, and functional assay results with experiments including non-tumorigenic cell lines and additional HCC cell lines. A focus of future assays can investigate NK cell production of IFN-γ and TNF-α in the presence of LLT1-blocked HCC cells. Additionally, subcellular fractionation and western blotting should be performed to confirm the location of LLT1 on and within the cell.

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CHAPTER III

INTERNSHIP SITE

My internship site was at the University of North Texas Health Science Center. My principal investigator was Dr. Stephen Mathew. The immunology lab focuses on the role of natural killer receptors in diseases, such as cancer. NK cells are the first line of defense against cancerous and virally infected cells, therefore further elucidating the roles of NK receptors in the context of anti-cancer immunotherapies is essential. Some of the other projects from the lab include the role of NK cells in the context of breast and prostate cancers, leukemia, and glioblastoma.

INTERNSHIP SUMMARY

My ultimate career-related goal is to work in translational science collaborating with teams of scientists and health care professionals to develop novel diagnostics and therapeutics for rare diseases. Prior to my internship, I had no experience in academic or cancer immunotherapy research. However, I had work experience at a large contract research organization (CRO) as a clinical research coordinator (CRC) and lab technician. Therefore, this past year (Summer 2022-Summer 2023) has held a tremendous number of learning opportunities.

Prior to experimentation, I had to become familiar with the background literature for HCC and basic immunotherapy concepts, including the complexities of tumor microenvironments, immunosurveillance, immune self-tolerance, and cancer evasion. Writing the research proposal was a great opportunity to learn about study design, different methodologies, and the general responsibilities required in graduate academic research.

Having such limited experience in research provided myriad opportunities for learning. For several months after writing my proposal, I spent the majority of my effort learning cell culture techniques. Next, it was important to ensure the presence of the protein of interest before investigating it's functional role. In order to evaluate mRNA expression, RNA had to be extracted from the cells. As RNA

is more likely to degrade in storage, it was essential to learn how to convert RNA to complementary DNA (cDNA). Then, PCR/gel electrophoresis (to detect the presence of LLT1) and qPCR (to determine relative mRNA expression among HCC cell lines) were performed. Because mRNA expression does not necessarily equate to surface expression, I learned how to perform flow cytometry. Flow cytometry uses lasers and fluorescence to investigate the chemical and physical characteristics of cells. Using conjugated monoclonal antibodies and stains, such as carboxyfluorescein succinimidyl ester (CFSE), I learned how to analyze a combination of tumor and natural killer cells for surface expression and apoptosis. I also gained experience with cellular fractionation, protein extraction, western blotting, and peripheral blood mononuclear cells (PBMC) isolation. Cellular fractionation, protein extraction, and western blotting is used to analyze each cellular portion (membrane, cytosol, and nucleus) for protein expression.

After investigating the cell lines for the presence of LLT1, the next step was to conduct functional assays. My first attempt was to perform a DELFIA-EuTDA assay. This assay involves loading target cells with bis-(acetoxymethyl)-2,2':6'2"-terpyridine-6,6"-dicarboxylate (BATDA). BATDA is hydrolyzed within the cell causing the molecule to become trapped within. As the cells lyse during incubation with NK cells, the percentage fluorescence released corresponds with percentage death, and thus a measurement of NK cell-mediated tumor lysis. However, attempts to perform this assay failed. Therefore, a new approach had to be designed. Using CFSE in a similar fashion as BATDA, tumor cells were tagged. Upon lysis during incubation with NK cells, CFSE was released which allowed for gating of HCC cells only using FlowJo software. When gating for $CFSE^+$ and PI^+ cells, this allows for distinguishing between the percentage of dead and alive HCC cells and NK cells. Additionally, using CD107a and CD56 (a NK cell surface marker) stains, I learned how to evaluate NK cell degranulation via tracking changes in CD107a (a functional biomarker for NK cell activity) across different experimental conditions.

Overall, my internship experience has been incredibly rewarding. I learned a tremendous amount on cancer immunotherapy, the academic research process, manual lab techniques, and so much more. Through seminars and interactions with other students and faculty, I have had opportunities to meet

incredible people, learn from their experiences, gain career insights, and, in general, grow in maturity and professionalism.