The Role of HHLA2 in NSCLC.

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Specific Aims

Lung cancer is the second most common cancer in the USA after prostate cancer in men and breast cancer in women. Each year, there are approximately a quarter-million new diagnoses, with the most prevalent diagnoses being non-small cell lung cancer (NSCLC).[7] Moreover, the disease causes approximately 160,000 deaths per year, making lung-cancer the leader in cancer-related deaths in the USA. This accounts for more deaths than breast, prostate, and colorectal cancers combined.[8] Prevention and awareness are crucial, as conventional therapies on early-stage patients have success in being cured. Conversely, stage III and IV lung cancer patients have a five-year survival rate of 5% and 1%, respectively.[7] More recently, the identification of different oncogenic drivers have improved this prognosis by allowing patients who harbor mutations in genes that drive tumorigenesis to be treated with small molecule inhibitors to halt pathway signaling of these oncogenic drivers. Particularly, patients who harbor mutations in the *EGFR* gene are predicted to have a response rate of over 80% to specific tyrosine kinase inhibitors.[9] Similarly, patients who harbor *ALK* translocations have a response rate of 50-60% to ALK inhibitors.[10] This era of improved bioinformatics has dissected NSCLC into different subsets based on their oncogenic drivers, making personalized medicine one of the forefronts of treating lung cancer. Despite these advancements, lung cancer patients who undergo personalized therapy often relapse through a variety of mechanisms including induction of alternate oncogenic pathways or immune evasion. As such, the immunotherapy via checkpoint blockade in NSCLC has also been heavily investigated and has proven to improve overall survival, such as with use of PD-L1, PD-1, or CTLA-4 blocking antibodies.[2, 6, 11] More recently, the ligand, named HHLA2, has been shown to be overexpressed NSCLC, but most prevalent in EGFR mutant NSCLC.[5] HHLA2 shows similarity to the B7 family of T-lymphocyte co-inhibitory molecules. Unlike the other members of the B7 family such as PD-L1,2/PD-1 or B7-2/CTLA-4, which have been studied extensively in human cancers and show clinical benefit in patients, HHLA2 is largely unstudied and its clinical significance to human lung cancer is unknown. Still, preliminary studies show that HHLA2 inhibits T-lymphocyte proliferation as well as cytokine production.[3] Thus, I hypothesize that targeting HHLA2 will have a therapeutic effect on NSCLC. In the tumor microenvironment of NSCLC, where immune evasion is a mechanism of cancer cell survival, it is important to understand the behavior of HHLA2 in terms of T-lymphocyte activation. Knowing the mechanism of action and the consequences of HHLA2's signaling pathway will provide a basic understanding of HHLA2's role in T-lymphocyte responses. ***Taken together, the central hypothesis of this study is that targeting HHLA2 in NSCLC will be effective for killing cancer cells through a cytotoxic T-cell mediated mechanism of action.***

To test the central hypothesis and achieve the objectives of the project, I propose the following two aims:

Aim 1: Analyze the role of HHLA2 on T-cells within the tumor microenvironment. Briefly, to test Aim 1, human biopsies of NSCLC will be stained by immunohistochemistry for HHLA2. Furthermore, the biopsies will be submitted to flow cytometry for characterization of HHLA2 expression and T-cell sub-populations. *In vitro* models of non- and high-expressing HHLA2 NSCLC with matched T-cells will also be developed to examine HHLA2's role in T-cell activation.

Aim 2: Determine the therapeutic efficacy of targeting HHLA2 in NSCLC. Broadly, to test Aim 2, *in vitro* models of non- and high-expressing HHLA2 NSCLC with matched T-cells will be developed with determine the therapeutic efficacy of targeting HHLA2 by treating them with either an HHLA2 blocking antibody or a control antibody. In addition, high-HHLA2 expressing NSCLC mouse models will be treated with an HHLA2 antibody or a control antibody, and observed for survival.

Accomplishing the aims of this project will provide valuable information about the role of HHLA2 in lung cancers. Elucidating the behavior of HHLA2 will reveal the mechanism by which the protein can lead to inhibition of T-lymphocytes that are necessary for cancer cell clearance. If the project is successful, the data will provide useful information to drug developers to create anti-bodies targeting the HHLA2 pathway for use in treating NSCLC and other cancers.

Background and Significance

There are approximately 200,000 lung cancer diagnoses annually, making it the second most common cancer in the USA after prostate cancer in men and breast cancer in women.[7] Astoundingly, lung cancer accounts for approximately 155,000 deaths each year, accounting for more annual deaths caused by breast, colon, and pancreas cancer combined.[8] Perhaps what makes lung cancer so deadly is that diagnoses usually occurs during stage III or IV, when the disease has progressed to an advanced metastatic state.[12] It is apparent that alternative therapies are necessary to improve this outcome. A single treatment option is impossible to account for all lung cancer patients due to complexity of lung cancer histology and heterogeneity. Lung cancer is divided into two main types including small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC accounts for 85% of all lung cancers, and can be further divided into three additional subtypes including adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. The most prevalent NSCLC subtype is adenocarcinoma, which will be the basis of this study.[13]

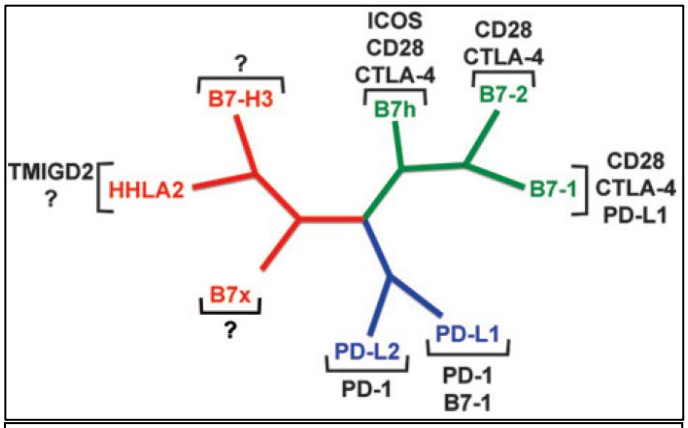

Figure 1: Phylogenetic tree of the human B7 and CD28 families. HHLA2, a newly identified member of the B7 family shares homology with well-known T-cell co-stimulatory or co-inhibitory molecules, B7-1, B7-2, B7h, PD-L1, and PD-L2, implying that HHLA2 will be an effective therapeutic target in cancer.[1]

NSCLC can be further divided into multiple subtypes based on their oncogenic drivers.[14] In particular, patients with driving mutations in the *epidermal growth factor receptor* (*EGFR*) or *anaplastic lymphoma kinase* (*ALK*) can therapeutically benefit from specific tyrosine kinase inhibitors (TKIs).[9, 10] In fact, screening for both *EGFR* and *ALK* is a routine practice in the clinic[15] because of the initial success seen in patients when treated with the TKIs. However, patients who do not harbor these mutations do not gain clinical benefit from the specific TKIs. Furthermore, patients who do harbor these specific oncogenic mutations and receive treatment, generally relapse within one year of treatment through resistance mechanisms employed by the cancer cells or immune cells of the tumor microenvironment.[16] Many efforts have been made to target these resistance mechanisms, and there are many second-line and third line specific inhibitors that target resistance of EGFR-TKI and ALK-TKI inhibition.[17, 18] While these resistance mechanisms can be specific and different from patient to patient, immunotherapy can address a majority of patients,

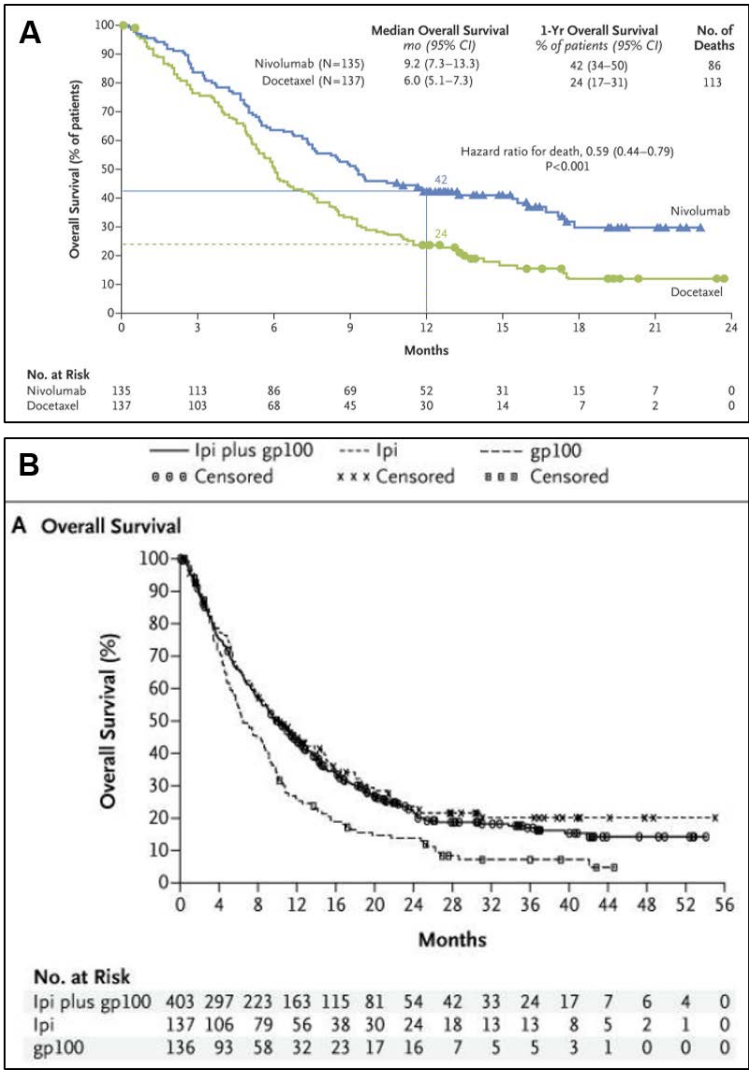

Figure 2: Checkpoint blockade via immunotherapy prolongs survival in lung cancer and melanoma. Figure 2A represents a Kaplan Meier survival plot of squamous NSCLC late stage patients who relapsed after platinum based chemotherapy that were treated with Nivolumab, a PD-1 blocking antibody, or Docetaxel, an anti-microtubule chemotherapy medication. The plot shows a significant increase in the 1-year overall survival in patients treated with Nivolumab compared to Docetaxel (42% vs 24%, hazard ratio=0.59, p<0.001).[2] **Figure 2B** represents a Kaplan Meier survival plot of patients with advanced unresectable melanoma that relapsed from standard chemotherapy. The plot shows a significant increased survival in patients who received Ipilimumab, a CTLA-4 blocking antibody, compared to gp100, a well-studied melanoma cancer vaccine (27.8 months vs 17.2 months, hazard ratio 0.68, p<0.001).[6]

rather than specific patients that harbor certain mutations. For instance, targeting the PD-1 pathway, which is a pathway of T-cell co-inhibition, has therapeutic benefit in patients with high and low expression levels of its ligand, PD-L1, in the tumor cells.[19-23] PD-L1 screening is also routine in patients who fail the first line chemotherapy treatment.[15] Similar to the specific TKIs, cancer patients can gain resistance to anti-PD-1 treatment.[24-26] Thus, there is an unmet need of alternative therapies for patients who relapse from initially beneficial drugs. Moreover, in the time where NSCLC seems to be more aggressive than ever, it is crucial to discover as many targets as possible to help the millions of patients affected each year by lung cancer.

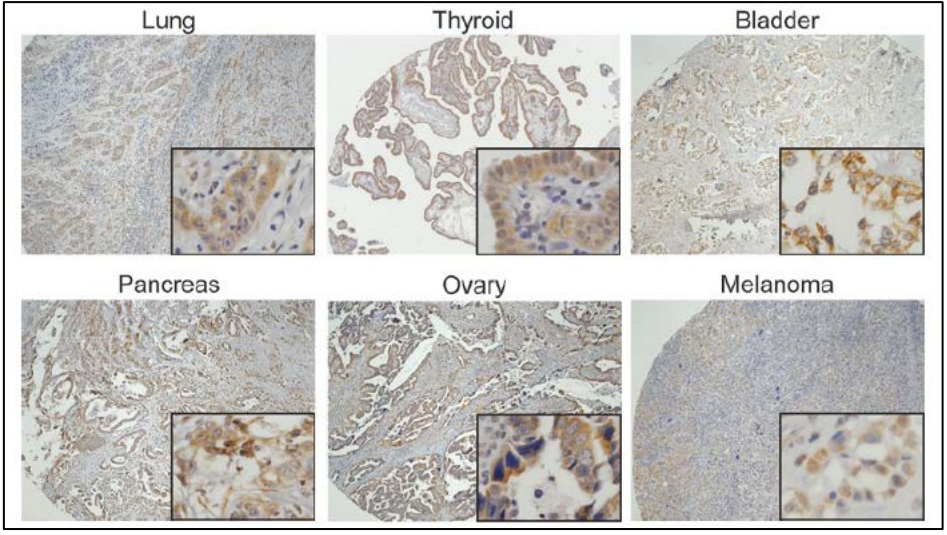

Figure 3: HHLA2 is widely expressed in various human cancers.
Immunohistochemistry staining of HHLA2 protein. HHLA2 was expressed in 50% or more in these cancer types. Images were acquired at 5x and 40x.[1]

One novel immune target worth exploring is HERV-H LTR-Associating 2 (HHLA2). HHLA2 has recently been described to be a member of the B7 family (**Figure 1**)[1], which are a group of ligands that can bind to T-cell co-inhibitory receptors.[3, 27, 28] These ligands include those that bind to known T-cell co-inhibitory receptors, PD-1 and CTLA-4. One receptor has been identified for HHLA2, called TMIGD2.[1] Studies suggest that another receptor exists for HHLA2 due to controversial results showing activation and inactivation of T-cells binding to HHLA2.[3, 28] Profound results using PD-1 and CTLA-4 blocking antibodies in many cancers including lung cancer and melanoma underscore the effectiveness of immunotherapy in cancer treatment.[2, 6, 11, 19, 21, 23] T-cell activation requires two signals, the first is engagement of the T-cell receptor with the MHC-peptide complex and the second is a co-stimulation signal provided by B7-CD28. The second signal can also be co-inhibitory. Engagement of the PD-1 and CTLA-4 receptors act as co-inhibition, and will thus prevent T-cell activation. PD-1 and CTLA-4 blocking antibodies work by reversing this T-cell inactivation.[29, 30] The impact of PD-1 and CTLA-4 blocking antibodies, termed immune checkpoint blockade, in the clinic is tremendous. In fact, the use of the PD-1 blocking antibody, Nivolumab, in a Phase 3 clinical trial in advanced squamous NSCLC, resulted in a significantly higher survival rate compared to patients who received docetaxel, an anti-mitotic/anti-microtubule chemotherapy medication (**Figure 2A**).[2] Inhibition of CTLA-4 is similarly impressive. In a Phase 3 Clinical Trial, patients with unresectable metastatic melanoma who failed first line chemotherapy received a survival benefit from ipilimumab, a CTLA-4 blocking antibody, compared to gp100, a well-studied melanoma cancer vaccine (**Figure 2B**). These data emphasize the immune tolerance of these cancers by the immune checkpoints, PD-1 and CTLA-4.[6] The clinical benefit using blockade of these immune checkpoints have created a revolution towards the discovery of many other types of immunotherapy targeted against cancer. This provides the enthusiasm and rationale to explore HHLA2 in the context of cancer treatment. HHLA2 expression is reported in many cancers including cancers of the breast, lung, thyroid, skin, ovary, and pancreas, but not in the normal cells of these organs (**Figure 3**). [1, 4] The expression of HHLA2 in many different cancers and not in normal tissue offers a clinical significance towards studying its role in tumorigenesis. Studies indicate HHLA2's essential role in T-cell suppression, and thus immune tolerance of tumors. Specifically, Zhao et al.

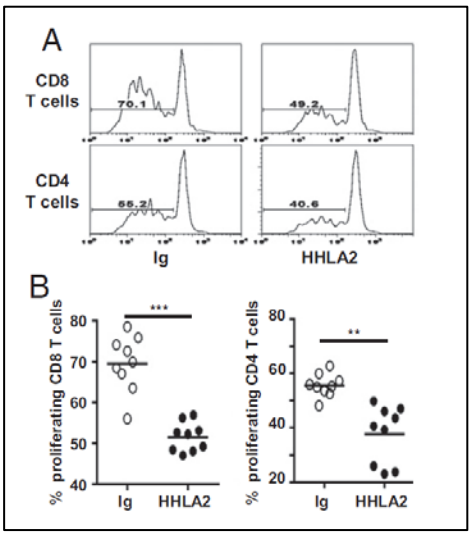

Figure 4: **HHLA2 decreases T-cell proliferation.** T-cells from PBMCs were cultured with anti-CD3 and a control antibody or an HHLA2 antibody. To measure proliferation, T-cells were labelled with CFSE. Upon proliferation, CFSE will split into the daughter cell, and will thus decrease. Results from the experiment shows a significant decrease in proliferation based on CFSE intensity (**Figure 4A**), quantified in **Figure 4B,** in both CD4 and CD8 T-cells when cultured with an HHLA2 antibody, suggesting its T-cell co-inhibitory function. [3]

reported a decrease in proliferation of T-cells from peripheral blood mononuclear cells (PBMCs) when cultured with anti-CD3 and an HHLA2 antibody compared to cells cultured with the control antibody (**Figure 4**).[3] The effect of T-cell suppression is an aggressive tumor; following this reasoning, HHLA2 expression was associated with metastases and a poor prognosis in osteosarcoma (**Figure 5**).[4] Still, the mechanism is which HHLA2 employs within the tumor microenvironment (TME) of any cancer is unknown. Of interest to our group, HHLA2 is significantly overexpressed in patients with the NSCLC adenocarcinoma subtype, which account for 40% of NSCLC cases. Cheng et al. reports that 69% of adenocarcinoma NSCLC patients express HHLA2 regardless of stage of disease, indicating that HHLA2 is worth targeting in these patients **(Table 1)**.[5] Furthermore, overexpression of HHLA2 is significantly associated with an EGFR mutant subtype in NSCLC, implying an alternative target in EGFR mutant and/or resistant cells (**Table 1**).[5] This is of significance to patients who relapse after treatment with specific EGFR TKIs. Data regarding the behavior of HHLA2 in the NSCLC tumor TME is relatively unknown, and understanding the mechanisms of HHLA2 activation will be crucial in understanding the implications of its overexpression in NSCLC and other cancers. Elucidating these mechanisms will provide valuable information about its relationship to T-cell activation and tumorigenesis.

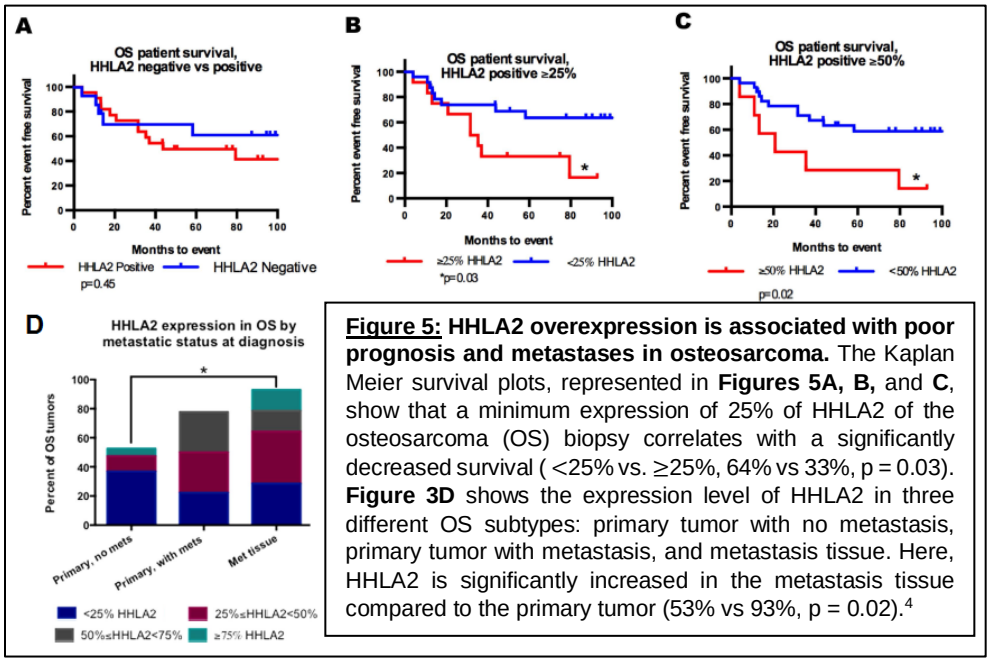

Figure 5: HHLA2 overexpression is associated with poor prognosis and metastases in osteosarcoma. The Kaplan Meier survival plots, represented in **Figures 5A, B,** and **C**, show that a minimum expression of 25% of HHLA2 of the osteosarcoma (OS) biopsy correlates with a significantly decreased survival (<25% vs. ≥25%, 64% vs 33%, p = 0.03). **Figure 3D** shows the expression level of HHLA2 in three different OS subtypes: primary tumor with no metastasis, primary tumor with metastasis, and metastasis tissue. Here, HHLA2 is significantly increased in the metastasis tissue compared to the primary tumor (53% vs 93%, p = 0.02).[4]

Based on the above-mentioned observations, the ***central hypothesis of this study is that targeting HHLA2 in NSCLCs will be effective for killing cancer cells through a cytotoxic T-cell mediated mechanism of action***. To test the central hypothesis and achieve the objectives of the project, I propose the following two specific aims: **1.)** *Analyze the role of HHLA2 on T-cells within the tumor microenvironment*. and **2.)** *Determine the therapeutic efficacy of targeting HHLA2 in NSCLC*. This information will be beneficial to drug creators in the development of HHLA2 anti-bodies as a novel immunotherapy in lung cancer and other cancers.

	Discovery cohort (n = 392)				Validation cohort (n = 287)		
Parameter	**HHLA2 Negative**	**HHLA2 Positive**	***P***	**Parameter**	**HHLA2 Negative**	**HHLA2 Positive**	***P***
Age, year	67.9	67.5	0.78	Age, year	70.5	70	0.47
Gender			0.60	Gender			0.09
Female (n = 215)	78 (36%)	137 (64%)		Female (n = 180)	46 (26%)	134 (74%)	
Male (n = 141)	55 (39%)	86 (61%)		Male (n = 80)	29 (36%)	51 (64%)	
Histology			<0.0001	Histology			0.92
Adeno (n = 290)	91 (31%)	199 (69%)		Adeno (n = 186)	58 (31%)	128 (69%)	
Squam (n = 31)	20 (65%)	11 (35%)		Squam (n = 29)	8 (27%)	21 (73%)	
Large (n = 18)	16 (89%)	2 (11%)		Large (n = 3)	1 (33%)	2 (67%)	
Stage			0.09	Stage			0.39
I (n = 252)	85 (34%)	167 (66%)		I (n = 157)	43 (27%)	114 (73%)	
II (n = 47)	23 (49%)	24 (51%)		II (n = 39)	15 (38%)	24 (61%)	
III (n = 35)	10 (29%)	25 (71%)		III (n = 22)	7 (31%)	15 (69%)	
Mutation status			0.04	Mutation status			0.01
EGFR (n = 41)	10 (24%)	31 (76%)		EGFR(n = 44)	5 (11%)	39 (89%)	
KRAS (n = 62)	23 (37%)	39 (63%)		KRAS (n = 66)	24 (36%)	42 (64%)	
WT/WT (n = 91)	43 (47%)	48 (53%)		WT/WT (n = 88)	27 (31%)	61 (69%)	

Table 1: Clinical and pathologic features of HHLA2 expression in patients with lung cancer. HHLA2 expression was explored in a lung cancer discovery cohort of 392 patients and a validation cohort of 387 patients. Clinical and pathological data was statistically correlated to HHLA2 negative and positive expression by immunohistochemistry. Results show that HHLA2 expression is most prevalent in lung adenocarcinoma compared to squamous cell or large cell carcinoma (p<0.0001). Furthermore, HHLA2 is expressed in all stages of lung cancer. Interestingly, HHLA2 expression is significantly associated with an EGFR mutational status (p=0.04). [5]

Research Approach

I hypothesize that HHLA2 functions as a T-cell co-inhibition molecule within the TME; blocking it within the TME of NSCLC will be therapeutically effective. To test this hypothesis and to specifically understand the role that HHLA2 plays in the TME of NSCLC, stage III or IV NSCLC biopsies will be gathered from the Thoracic Head and Neck Medical Oncology Department at the MD Anderson Cancer Center. The utility of the biopsies will be two-fold. First, analysis of the overall T-cell nature of the TME based on HHLA2 expression will be performed by flow cytometry. Second, I will develop an autologous model system whereby NSCLC cell lines and their T-cells will be expanded for functional assays and *in vivo* humanized mouse studies.

Aim 1: Analyze the role of HHLA2 on T-Cells within the tumor microenvironment.

1.1 Analyze the T-cells of the TME in NSCLC based on HHLA2 expression.

Zhao et al. reported that HHLA2 can inhibit the proliferation of both CD4 and CD8 T-cells. In this study, an *in vitro* model was used whereby CFSE-labelled T-cells were cultured with plate-bound anti-CD3 and a control antibody or an HHLA2 antibody (**Figure 4**)[3]. Although this experiment reported inhibition of proliferation of T-cells by HHLA2, the role of HHLA2 in the context of tumor expression is lacking. First, to determine the expression level of HHLA2 in the NSCLC samples, a portion of the NSCLC biopsy will be submitted to HHLA2 immunohistochemistry (IHC), real-time PCR, and western blot. The biopsies will be analyzed and scored based on the percentage of HHLA2 positive cells via IHC. Specifically, 0% is no expression (score 1), 1-15% is low expression (score 2), 16-30% is moderate expression (score 3), and 31+% is high expression (score 4). The scoring will be performed by two blinded pathologists. The scores will be validated by real-time PCR and western blot. Normal human lung fibroblasts from willing donors will be used as a negative control. I hypothesize that more than 50% of the patients will have an HHLA2 expression score of 3 or 4, based on previous studies conducted by Cheng et al. These data will be correlated with the flow cytometry data described below. To discover how HHLA2 influences T-cell responses in the TME, these biopsies will be processed into single cell suspensions using standard tumor processing protocols for flow cytometry experiments. The cells will be stained for HHLA2 (doing so will provide validation of the IHC, real-time PCR, and western blot data) and other markers to characterize the different T-cell populations, including those for cytotoxic T-cells (CD8, Granzyme B), proliferative/active T-cells (CD4, CD8, CD38, and Ki67), exhausted T-cells (CD4, CD8, PD-1, TIM-3, LAG-3), and regulatory T-cells (CD4, CD25, and FOXP3). Generation of data will be performed on the BD FACS Celesta flow cytometer, which can detect up to 14 parameters. The data will be analyzed using the flow cytometry analysis software, FlowJo. The final data will be undergo statistical analysis for determination of any correlation between over- or underrepresentation of particular T-cell subtypes and HHLA2 expression in tumor cells. Considering the fact that HHLA2 comes from the same family as the ligands that engage PD-1 and CTLA-4, it is expected that a high expression of HHLA2 will be associated with low levels of proliferative/active T-cells and high levels of exhausted T-cells. Exhausted T-cells have been reported to be maintained by regulatory T-cells[31]; thus, I also expect a high expression of HHLA2 will be associated with high levels of regulatory T-cells.

For conducting further functional studies, I will develop models of human NSCLC cell lines and matched T-cells from the biopsies. Two small (1 mm) sections of the biopsies will be cut and cultured separately for tumor cell expansion and T-cell expansion. The tumor cells grow in complete RPMI media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The T-cells grow in the same medium supplemented with 6000 units of IL-2; these cells can be further expanded using the rapid expansion protocol, which have been previously published.[32-34] Once the models are developed, the different cell lines will be screened for HHLA2 expression by real-time PCR and western blot to be validated with results from the tumor sample itself, mentioned above. To test the role of HHLA2 on T-cells, the tumor cells will be cultured with CFSE-labelled matched T-cells to measure proliferation. Proliferation can be monitored by levels of CFSE. The T-cells can also be screened for a CD4 or CD8 phenotype through staining by flow cytometry. The T-cells will also be stained for the different T-cell sub-populations as mentioned above to understand the direct causes of HHLA2 on cytotoxic, proliferative/active, exhausted, and regulatory T-cells. I expect proliferation of the CD4 and CD8 cells to be low, when cultured with tumor cells that have high HHLA2 expression. Furthermore, a low HHLA2 tumor expression pattern will be associated with high levels of cytotoxic and proliferative/activated T-cells and low levels of exhausted T-cells and low regulatory T-cells. I must also consider effects caused by PD-L1, which has also been reported to be highly expressed in NSCLC. If complications arise, I will also looking at PD-L1 expression in these cancer cell lines to elucidate any correlations between PD-L1 and HHLA2 expression and its role in T-cell responses. Understanding these mechanisms will help clarify roles of T-cell exhaustion and inactivation in terms

of cancer clearance and HHLA2 expression, which will be necessary in drug development and the subsequent determination of treatment decisions in patients with NSCLC.

1.2 Examine the changes of T-cell sub-populations when HHLA2 is blocked within the TME.

To test T-cell responses in the absence of HHLA2, the human NSCLC cancer cells and matched T-cell models will be cultured with anti-CD3 and a control antibody or an HHLA2 blocking antibody (to be developed in-house). To have translational significance, blocking HHLA2 should show an increase in cytotoxic T-cell proliferation and activation, which should override any increases in regulatory T-cells or exhausted T-cells to kill cancer cells. Through culturing high-HHLA2 expressing NSCLC cells with their matched T-cells with and without an HHLA2 blocking antibody, changes in T-cells can be tracked by flow cytometry using the markers mentioned in **Aim 1.1**. I expect that when the high-HHLA2 expressing NSCLC cells and their matched T-cells are cultured with anti-CD3 and an HHLA2 blocking antibody, there will be an increase in proliferative/active and non-exhausted T-cells (both CD4 and CD8), similar to the levels seen in the samples of non-expressing HHLA2 NSCLC. It is possible that exhaustion by PD-1 or CTLA-4 signaling may occur, if this is the case, a PD-1 blocking antibody (Nivolumab) and a CTLA-4 blocking antibody (Ipilimumab) will also be tested as single treatment agents and in combination with the HHLA2 blocking antibody; *in vivo* models will be sought out as necessary.

1.3 Investigate the activation of tumor infiltrating lymphocytes (TILs) and splenocytes of cancer-bearing treated mice.

One difficulty that comes with studying HHLA2, is that it is expressed in humans but not in mice. To overcome this hurdle, an *in vivo* humanized mouse model will be used. In this model, the highly immunodeficient NSG mice are irradiated, and receive an injection of CD34+ hematopoietic stem cells from human cord blood (**Figure 6**). This mouse can support engraftment of different myeloid and lymphoid lineages. In addition, this mouse model allows for immune cell homing of all immune organs.[35, 36] In this study, one major advantage of this model is that a human cell line can be engrafted and successfully grow due to the immunodeficient NSG background. Thus, I can orthotopically inject within the mouse, the developed NSCLC cell lines that have known HHLA2 expression. Therefore, I can develop a mouse model of non- and high-HHLA2 expressing tumors *in vivo*. These implanted mice will then be treated either with a control blocking antibody or an HHLA2-blocking antibody and will be used for functional and survival studies. Preliminary studies will be conducted to determine the degree of growth for these NSCLC cell lines; doing so will help to determine which day will be appropriate to start treatment.

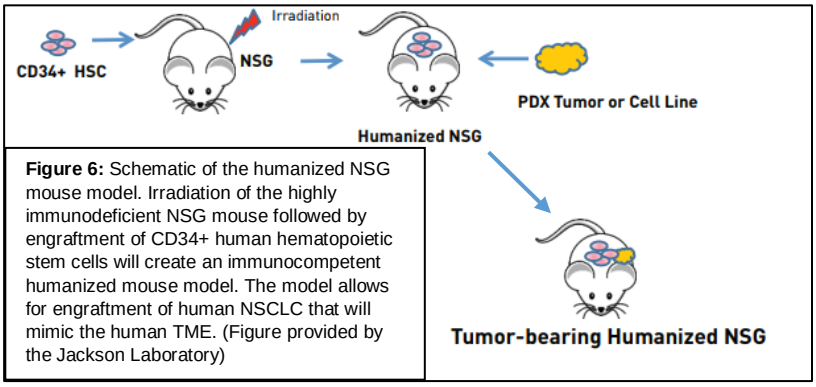

Figure 6: Schematic of the humanized NSG mouse model. Irradiation of the highly immunodeficient NSG mouse followed by engraftment of CD34+ human hematopoietic stem cells will create an immunocompetent humanized mouse model. The model allows for engraftment of human NSCLC that will mimic the human TME. (Figure provided by the Jackson Laboratory)

In an effort to confirm my hypothesis that blocking HHLA2 will cause cytotoxic T-cell activation, I will conduct *in vitro* co-culture experiments, where the target cells, which include non-and high-HHLA2 expressing cells, will be cultured with TILs or splenocytes from the mice that are differentially treated cancer-bearing mice (control antibody or HHLA2 blocking antibody). ELISAs will be used to detect levels of different T-cell activation cytokines, including IFNγ and IL-2. I expect the highest levels of IFNγ and IL-2 in the cells co-cultured with TILs or splenocytes from the mice treated with the HHLA2 blocking antibody.

Aim 2: Determine the therapeutic efficacy of targeting HHLA2 in NSCLC.

2.1 Test the therapeutic effect of a blocking HHLA2 in NSCLC.

Given that HHLA2 is highly expressed in NSCLC and has been shown to hinder T-cell activation[3], I hypothesize that blocking HHLA2 will reverse T-cell inactivation and be effective in killing tumor cells. First, to test the therapeutic effect of targeting HHLA2 in NSCLC, the human NSCLC cancer cells and matched T-cell models will be cultured with anti-CD3 and a control antibody or an HHLA2 blocking antibody. Cell proliferation and viability of the cancer cells will be measured using the Vi-CELL™ Cell Viability Analyzer to count viable cells after treatment. In addition, cell proliferation of cancer cells will be measured using the CyQUANT Cell Proliferation Assay Kit, which will provide proliferation information by virtue of cellular DNA amount. Furthermore, to test cell death, the Annexin assay will be utilized. The models will be grouped into non- and high-HHLA2 expressing NSCLC. I hypothesize that the high-HHLA2 expressing cancer cells cultured with the HHLA2 blocking antibody will undergo less proliferation and more apoptosis compared to the control antibody group.

To test the therapeutic efficacy of blocking HHLA2 *in vivo*, the humanized mouse model, as mentioned in **Aim 1.3** will be used. The developed non- and high-HHLA2 expressing NSCLC tumor cell lines will be orthotopically injected and subcutaneously injected within the flank. The mice will be split into two groups, one treated with a control antibody and the other with the HHLA2 blocking antibody. Signs of toxicity, including hunched back, lethargy, waddling, and diarrhea, will be monitored to track survival. The flank tumor will also be measured to track tumor growth. I hypothesize that the control mice, injected with the non-HHLA2 expressing tumor cells, will have a slowed tumor growth and a delayed death compared to mice injected with the high-expressing HHLA2 tumor cell line. In addition, mice treated with the HHLA2 blocking antibody will have an extended survival, slowed tumor growth, with the possibility of long-term survivors compared to the control mice.

2.2 Investigate the synergy of blocking HHLA2 and PD-L1 in vivo.

Screening for PD-L1 in NSCLC patients is routine.[15] Dramatic responses have been reported in lung cancer patients that are treated with either a PD-1 or PD-L1 antibody. PD-L1, PD-L2, and HHLA2 come from the same family and are identified as co-inhibitory molecules for T-cell inactivation. Because HHLA2 has recently been discovered, very little is known about its implications in NSCLC. Because it is highly expressed in NSCLC and is a protein of co-inhibition, it should be a good target for treatment with PD-L1 blocking antibodies. To investigate the synergy of HHLA2 and PD-L1 blocking antibodies, the humanized mouse model, as mentioned above, will be used. Non- and high-HHLA2 expressing orthotopic mouse models will be used and split into four different treatment groups. These treatment groups include 1) a control antibody, 2) a PD-L1 blocking antibody, 3) an HHLA2 blocking antibody, and 4) combined PD-L1 and HHLA2 blocking antibodies. I hypothesize that targeting HHLA2 and PD-L1 will show drug synergism through increased survival in both non- and high-HHLA2 tumor mouse models compared to the other treatment groups. It is feasible that PD-L1 may not be highly expressed on the cells, but may later arise due to anti-HHLA2 resistance. Thus, treatment with both the anti-HHLA2 antibody and the anti-PD-L1 antibody may not lead to increased survival compared to the single-treatment agents. The vice versa situation may also be true, and will also be considered. If PD-L1 upregulation is not an inherent immune evasion mechanism in this case, resistance to anti-HHLA2 may have to be produced; in this case, I will try sequential treatment options where anti-PD-L1 therapy will be followed by anti-HHLA2 therapy.

2.3 Inspect synergy of treatment using EGFR TKIs and an HHLA2 antibody in EGFR mutant NSCLC in vivo.

Cheng et al. recently reported that HHLA2 is widely expressed in NSCLC with a significant prevalence in patients with an EGFR mutational status. NSCLC patients who harbor driving mutations in EGFR initially have a therapeutic benefit from anti-EGFR tyrosine kinase inhibitors. However, most patients eventually relapse within a year because the anti-EGFR therapy becomes ineffective. Thus, it is important to find alternative therapies for patients with EGFR mutations, which account for about 25% of lung adenocarcinoma patients. To investigate the synergy of HHLA2 and PD-L1 blocking antibodies, the humanized mouse model will be used. For this experiment, the developed NSCLC cell lines will be screened for EGFR by real time PCR and western blot. Non- and high-EGFR expressing orthotopic NSCLC tumor cell lines will be used. The mice will be split into four different treatment groups including, 1) control, 2) HHLA2 blocking antibody, 3) EGFR TKI (Gefitinib), and 4) HHLA2 blocking antibody and Gefitinib. I hypothesize that the life of mice treated with Gefitinib and the HHLA2 blocking antibody will be extended compared to the other groups based on the theory that HHLA2 is highly expressed on EGFR mutant cells for tumor evasion. It is possible that HHLA2 may inactivate T-cells in response to EGFR TKI resistance. If this is the case, the combination treatment may be comparable to EGFR TKI treatment alone. If this is the case, an EGFR-TKI resistant model may have to be developed. Alternatively, sequential dosing may be administered whereby HHLA2 blockage may follow EGFR TKI treatment.

Alternative Strategies

Accomplishing these aims will provide valuable information about the effects of blocking HHLA2 to achieve an anti-tumor immune response. That is, the flow cytometry data will elucidate any changes in the TME in response to blocking HHLA2, which may provide the rationale to bring an HHLA2 blocking antibody to the clinic to provide alternative treatment options in NSCLC. If targeting HHLA2 is not ideal, I have also considered using an anti-body against its receptor TMIGD2. Other members of the B7 family, including B7x or B7-H3, has not been extensively studied in lung cancer, and would be worth investigating if HHLA2 blockage does not lead to anticipated results. I do not anticipate any technical or methodological problems in these mouse survival studies. In summary, the studies proposed in this project should demonstrate the benefit of targeting HHLA2 in NSCLC. If successful, this proposal will provide a strong rationale to support the clinical investigation of this strategy.

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