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Can anti-PD1/PD-L1 Immunotherapy Promote Macrophage Activity against HCC Tumor Cells?

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ABSTRACT

With over 750,000 new cases identified annually, hepatocellular carcinoma (HCC) continues to be a threat, with current immunotherapies proving ineffective in most patients. PD1/PD-L1 antibodies have risen as another treatment option, but previous studies have only analyzed these antibodies in the tumor microenvironment. In order to understand how exactly these PD1/PD-L1 blockers affect macrophages and their anti-tumor macrophages, this study isolated the macrophage cells and tumor cells. Two protocols were followed, one with the B16F10 melanoma cell line, in order to determine proper procedures and determine a point of comparison, and one with the R1LWT liver cancer cell line. In the end, the B16F10 cell line responded positively to the PD1/PD-L1 antibodies, with increased MHCI/MHCII activation. On the other hand, the R1LWT cell line reacted oppositely, opening new inquiries into other pathways by which immunosuppression occurs.

Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer, with over 750,000 new cases identified annually, and current treatments having many limitations (Tian 2019). It is highly connected to inflammation, with various risk factors contributing to chronic inflammation in the liver. As a result, the resulting cycle of liver death and regeneration contributes to the progression of HCC (Huang 2021). Even with localized HCC and consequent radiation or resection, 70% of patients have tumor recurrence within 3 years (Yoo 2017), because the tumor microenvironment contains ongoing stress and an immunosuppressive atmosphere. Thus, effective immunotherapies are crucial to targeted treatments and anti-tumor immunity. This study will be analyzing the effects of PD1/PD-L1 antibodies in HCC cells, quantified by MHCI and MHCII activation within the co-culture of macrophages and tumor cells.

Macrophage Role in Tumor Development

Macrophages play a crucial role in the innate immune system, as they perform phagocytosis on pathogens and emit antimicrobial mediators. They are monocytes that have differentiated from myeloid cells- blood cells originating from the bone marrow (Hirayama 2017). As shown in Figure 1, macrophages present antigens that can be recognized by T-cells, resulting in immune responses against pathogens (Ovchinnikov 2020).

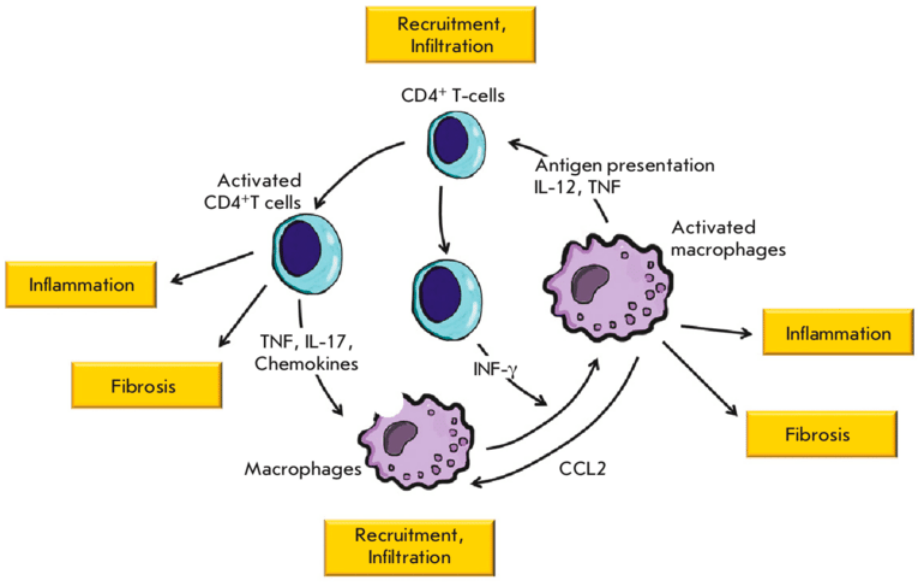

Figure 1: T cell activation by macrophages

They are thus involved in anti-tumor activity, but have been observed to contribute to the tumor microenvironment upon interaction with tumor cells. Monocytes activated by tumors express PD-L1 proteins, which suppress tumor-specific T-cell immunity (Kuang 2009). As proposed by Stout et. al, macrophages have functional adaptivity, in which they not only adopt different functional patterns in order to match the environment, but can change their phenotype in response to external signals (Stout 2005). This is exactly how they can play a dual role in the tumor microenvironment, with some macrophages participating in anti-tumor activity and others supporting tumor proliferation.

MHCI and MHCII

MHC (major histocompatibility complex) class I and II proteins are crucial for adaptive immunity, a subsystem in which specialized immune cells destroy foreign invaders and develop a memory for similar future pathogens (National Cancer Institute). Essentially, they are responsible for presenting peptides on the cell surface for recognition by T cells. MHCI -on the surface any cell with a nucleus- is recognized by cytotoxic CD8+ T cells while MHCII is presented on anti-presenting cells such as dendritic cells, macrophages, and B cells for recognition by CD4+ T cells (Wieczorek 2017). In both circumstances, MHCI and MHCII allow these T cells to arrange a response against invaders.

PD-1 and PD-L1

Programmed cell death ligand (PD-L1) is a cell surface glycoprotein expressed on tumor cells and by antigen-presenting cells (APCs), such as macrophages and dendritic cells. These molecules hinder the immune system's response, allowing the tumor to proliferate. Specifically, upon ligation by PD-L1, the programmed cell death protein (PD-1) inhibits T-cell growth and T-cell effector functions (Kuang 2009). This leads to the downregulation of the anti-apoptotic molecule Bcl-xl, cytokine expression, and the mTOR pathway in immune cells. Furthermore, in response to inflammation of the tumor microenvironment and tumor antigen expression, the induction of PD-1 and ligation by PD-L1 works to subside the immune response to HCC, creating a cycle of immunosuppression (Zhang 2019).

While PD-L1 checkpoint inhibitors have surfaced as an effective HCC treatment, only a minority of patients benefit. In reality, only 20-30% of patients experience a positive outcome from PD-1/PD-L1 blockade and primary resistance could eventually lead to HCC progression in clinical patients. As tumors are heterogeneous and cancer cells

can learn to evade PD-1/PD-L1, results vary across the numerous cancers. Thus, these antibodies still need to be researched extensively to fully understand the complexities of immunotherapy (Sun 2020). Furthermore, data has been found to indicate that treating macrophages with anti-PD-L1 antibodies can raise anti-tumor activity (Jilkova 2019). Essentially, anti-PD1/PD-L1 antibodies block the ligation of PD-1 by PD-L1, discontinuing the pathway that inhibits T-cell function. Figure 2 demonstrates this mechanism between the macrophage (CD8) and the tumor cells.

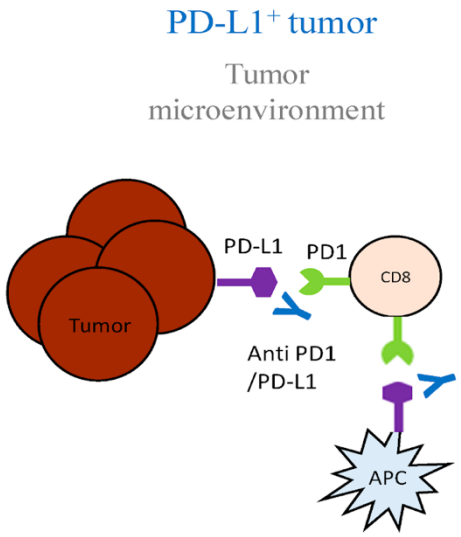

Figure 2: The mechanisms of anti-PD1/PD-L1 antibodies

Still, as the correlation between the antibody and upregulation of macrophage activity is uncertain, this project will be focused on measuring whether anti-PD1/PDL-1 immunotherapy can advance anti-tumor immunity against HCC cells by measuring MHCI and MHCII activation.

This leads to the question: Can anti-PD1/PD-L1 immunotherapy directly promote macrophage activity against HCC tumor cells? By studying macrophage activity in the presence of antibodies versus without, the exact interaction between macrophage upregulation and the progression of HCC tumor cells can be determined.

Literature Review

The Inflammatory Microenvironment in Hepatocellular Carcinoma: A Pivotal Role for Tumor-Associated Macrophages

The tumor microenvironment plays a crucial role in the development of hepatocellular carcinoma, especially regarding the inflammatory response. Providing an overview of this tumor microenvironment are Capece and others in the Department of Biotechnological and Applied Clinical Sciences at the University of L'Aquila. They conveyed how the microenvironment consisted of stromal cells, hepatic stellate cells, endothelial cells, growth factors, inflammatory cytokines, and extracellular matrix proteins. Tumor-associated macrophages (TAMs) produce signals that promote tumor growth, causing angiogenesis, metastasis, and poor prognosis. This study also expands upon the functional adaptivity aspect of macrophages by diving into TAM plasticity- macrophages in the peritumoral stroma are quickly activated against the tumor cells, but those in close proximity to cancer cells are exhausted and fail to advance the immune response. The activated macrophages in the peritumoral stroma have high expression of HLA-DR, Interleukin beta 1, Interleukin 6, and Interleukin 23, while the ones in the cancer nests express an HLA-DR-IL-10 phenotype.

Meanwhile, in humans, HCC cells have been shown to recruit macrophages through Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), TGF-beta, CCL2, or M-CSF. Consequently, the HCC cells facilitate immunosuppression, preventing phagocytosis and tumor death.

Activated Monocytes in the peritumoral stroma of HCC foster immune privilege and disease progression through PD-L1

Kuang and other researchers at the Department of Immunology in Sun Yat-Sen University qualify this focus on the HCC tumor microenvironment as they concentrate on the interaction between macrophages and other monocytes within (Kuang 2009). Some macrophages express surface PD-L1 that when activated, can suppress tumor-specific immunity and help tumor proliferation. Soluble factors in tumor cells trigger the activation of cytokines such as TFN-a and IL-10, which leads to the activation of PD-L1 and impairs anti-tumor cell immunity. Another significant contribution made by this study was the revelation that tissues from more advanced stages of HCC express higher levels of the PD-L1 protein.

Animal Models of Hepatocellular Carcinoma: The Role of the Immune System and Tumor Microenvironment

Moreover, such exacerbated PD-L1 expression in HCC cells inhibits the function of T cells in the liver tumor microenvironment. In the study done by researchers Macek-Jilkova, Kurma, and Decaens at the Institute for Advanced Biosciences in France, the correlation mentioned beforehand between high PD-L1 expression on tumor cells and the recurrence of HCC was confirmed (Macek-Jilkova 2020). Furthermore, this study explains the PD-1 and PD-L1 checkpoint inhibition and how existing drugs have shown promising response rates with this immunotherapy. Anti-PD-1 drug Keytruda has seen a response rate of 17% and anti-PD-1 antibody nivolumab (Opdiva) was recently implemented in successful trials. Yet, even with these clinical outcomes, anti-PD-1/PD-L1 blockers continue to be inefficient in 80% of HCC patients.

IL-6 promotes PD-L1 expression in monocytes and macrophages by decreasing protein tyrosine phosphatase receptor type O expression in human hepatocellular carcinoma

Zhang and others in the Department of Hepatobiliary Surgery at the Nanjing University Medical School attempt to further explicate intensified PD-L1 expression as they linked it to a decrease in protein tyrosine phosphatase receptor type 0 expression (PTPRO). According to this study, the JAK/STAT signaling pathway is what relates this decreased expression of PTPRO to raised PD-L1 (Zhang 2019). Additionally, IL-6 antibody combined with anti-PD-L1 antibodies has achieved significant results. Thus, decreased expression of PTPRO in TAMs causes exacerbated T cell exhaustion within the tumor. While this represents the relationship between PTPRO and the activation of PD-L1 in the tumor microenvironment, clinical studies still need to prove the exact interaction between macrophages treated with and without antibodies and the HCC cells in this microenvironment.

Research Question

As explained above, many research studies have researched the effects of anti-PD-1 and PD-L1 antibodies on tumor proliferation and have shown that macrophage activity results in anti-tumor activity. However, little is known about how immunotherapy acts directly on macrophages. Thus, by observing the fluorescence of the HCC tumor cells, we can look at the phagocytic activity of macrophages treated with and without the antibodies. This will exhibit exactly

how the antibodies either have an effect or not on macrophage activity against tumor cells. In fact, according to Yunzhou Pu and her article Tumor-Associated Macrophages Regulate PD1/PDL1 Immunosuppression, more convincing clinical studies need to correlate macrophage activity with tumor suppression when treated with antibodies (Pu 2022). Furthermore, a study done by Xu et. al even mentions how there is existing research about the complexities of the tumor microenvironment, but there are rarely any reports on the underlying mechanisms and interactions between the TAMs and HCC cells (Xu 2022). Moreover, the tumor microenvironment has many outside stressors and stromal cells that could play a role in the upregulation of T cells. In order to fully confirm that the antibodies are helping the macrophages increase anti-tumor activity instead of diminishing the immune response, more data needs to be established.

That is exactly what this project will be doing by isolating macrophage and tumor cells in order to ensure that the tumor microenvironment doesn't play a role in immune regulation. No other factors would be present, so any interaction between macrophages and tumor cells in the presence of anti-PD-1/PD-L1 antibodies versus without would confirm the previous conclusions of these underlying mechanisms.

Methods

Protocol 1 (B16F10-GFP-OVA Melanoma Cells)

To confirm previous results and establish proper protocols, experiments involving PD1/PD-L1 antibodies were first performed on melanoma cells. The B16F10-GFP cell line derived from mice was used for these initial experiments because research already exists on how these cells respond to antibodies (Shengqing 2021). This line also possesses PD1/PDL-1 pathways, so it can be reasonably inferred that the antibodies would increase MHC-I and MHC-II expression in these cells. This specific cell line also expressed GFP, meaning the live cells fluoresced when read by the flow cytometer.

The macrophage cell line J774A.1 was used with both the melanoma cells and the HCC cells, as it is a commonly used mouse model when studying the effects of antibodies and plays a key role in the phagocytosis of B16F10 cells (Erminia 2018). This line was gifted by Dr. Januario Castro at Mayo Clinic Arizona. After splitting the cells, both cell lines were incubated at 37°C and 5% CO_2 and were split/grown throughout the timeline of experiments.

Preparation of a Single Cell Suspension

B16F10-GFP cells

The flask of B16F10 cells was first washed twice with PBS (remove media, add 10 mL of PBS, discard, and repeat) and then 1 mL of 0.05% trypsin-1 mM EDTA was added to release the cells from the flask. After incubation at 37°C for 2 minutes, 4 mL of complete medium (DMEN 10% FBS) was added to stop trypsinization. The cells were triturated with a 5 mL pipette and were transferred to a 15 mL conical tube.

J774A.1 cells

The media was removed and the dish was washed twice with PBS, upon which 2 mL of Versene was added and the cells were incubated at 37°C for 10 minutes. After adding 3 mL of complete medium, the cells were triturated thoroughly to detach the cells, which were then transferred to a 15 mL conical tube.

Both tubes were centrifuged at 1200 RPM for 5 minutes at 4°C to pellet down the cells. Then the supernatant was discarded, the cells were resuspended in 2 mL of complete culture medium, and both the B16F10 and J774A.1 cells were counted under a microscope. A solution with a concentration of 2,000,000 cells/20 mL was prepared for each cell line utilizing a 50 mL conical tube.

Seeding the Cells in Co-Culture

The macrophage/tumor cells and PD1/PD-L1 antibodies were distributed according to the plate map below. The concentration of the cells was 10^5 cells/mL with 1 mL of each cell line in each of the wells (to form a 1:1 ratio for the co-culture). The 2nd plate acted as a control, as it only had macrophages. If MHCI/MHCII activation remained low in the samples of this plate, it would confirm our claim that MHCI/MHCII activation only increases when tumor cells are present. The cells were then incubated for 48 hours at 37°C and 5% CO_2 to prepare for flow cytometry.

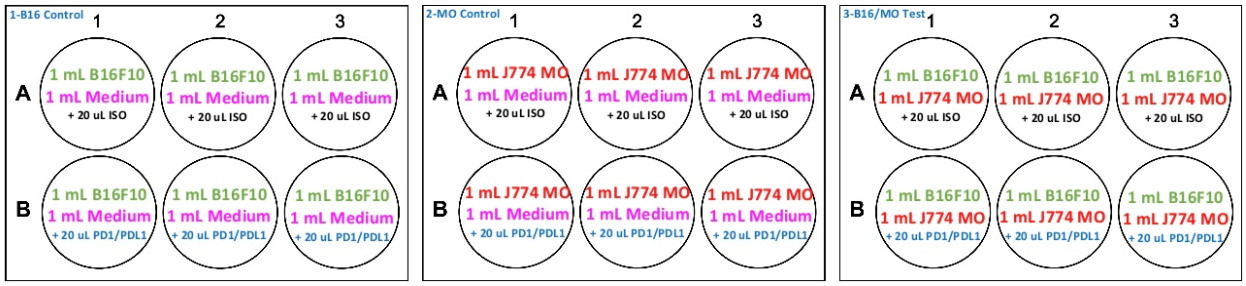

Figure 3: Plate Map for all experiments with B16F10 cells

Flow Cytometry Procedure

FACS tubes were identified with numbers correlated to those on each plate. For each well, the supernatant was collected into the previously identified FACS tubs and then washed twice with PBS (added 2 mL of warm PBS, aspirated the liquid, then repeated). 500 uL of warm Versene was added to each well to detach the cells and incubated for 10 minutes at 37°C. The cells were flushed to completely detach them, and the supernatant was collected into the same FACS tube. The cells were kept in ice until all the tubes were finished and then were centrifuged at 1500 RPM for 5 minutes at 4°C. The supernatant was discarded (the tubes were set upside down over a paper towel to ensure all of the remaining liquid was removed). The mix of antibodies and single-color controls to stain the cells was prepared according to the tables below (Figure 4). 50 uL of the antibody mix was then added to each tube, including those with the beads. The cells were incubated a minimum of 20 minutes in ice and were protected from the light. 2 mL of PBS was added to the tubes, and they were centrifuged before the supernatant was discarded and the cells were resuspended in 300 uL of PBS. The samples were finally acquired at the flow cytometer (FACSymphony™ A3) through the following channels.

Table 1: B16F10 Antibody Panel

ANTIBODY PANEL			
CHANNEL	**MARKER**	**COLOR**	**Antibody MIX (650 uL)**
B515/20	B16 GFP	GFP	-
YG 586/15	MHC-II	PE	3 uL
YG 610/20	Viability	ZombieRed	1 uL
R 670/30	MHC-I	APC	3 uL
R 780/60	CD45	APCy7	3 uL
SINGLE COLOR (Beads)			
CHANNEL	**MARKER**	**COLOR**	**Antibody**
Unstained	-	-	

B515/20	B16 GFP	GFP	CD4 FITC
YG 586/15	MHC-II	PE	MHC-II PE
YG 610/20	Viability	ZombieRed	Ly6C PE-CF594
R 670/30	MHC-I	APC	MHC-1 APC
R 780/60	CD45	APCy7	CD45 APCy7
EXTRA CONTROLS			
CHANNEL	**TUBE**	**COLOR**	**FUNCTION**
Unstained	J774 FRIDGE	UN	FLUO. BACKGROUND
B515/20	B16 GFP FRIDGE	**GFP**	FITC BACKGROUND

Protocol 2 (R1LWT Hepatocellular Carcinoma cells)

After the results of the first series of experiments with B16F10 melanoma cells and the protocols were established, the R1LWT mouse model was split. These HCC cells were derived from the RIL-175 cell line, which was isolated from hepatic tumors in C57BL/6 mice (Kapanadze 2013). The HCC cells were incubated alongside the macrophage cell line J774A.1 at 37°C and 5% CO_2 throughout the duration of the experiments. The cells were generously gifted by Dr. Dan G. Duda at Massachusetts General Hospital.

Preparation of a Single Cell Suspension

This protocol was the same as that of the B16F10 cells, except the melanoma cell flask was replaced with R1LWT. Before finishing the solution of the R1LWT cells, however, a CTV labeling procedure had to be completed.

Cell Trace Violet 405 nM Labeling Procedure for Proliferation Assay:

As the R1LWT cell line had no fluorescence, unlike the B16F10-GFP cells, CTV labeling was necessary to provide fluorescence. To administer CTV, a 5uM working solution was first created by diluting 2 uL of 5 mM CTV stock solution in 2 mL PBS (1 uL of CTV from the stock was used for every mL of cell suspension). After spinning down the R1LWT cell suspension solution containing 2,000,000 cells (1500 RPM at 4°C for 5 minutes), they were resuspended in 2 mL of the CTV working solution (1 mL was used for 1,000,000 cells). The cells were incubated for 20 minutes at 37°C while being protected from light. The staining was quenched by adding 10 mL of DMEN 10% FBS and then the cells were pelleted and resuspended in 20 mL of pre-warmed cell culture medium.

Seeding the cells in Co-Culture

The macrophage/tumor cells and PD1/PD-L1 antibodies were distributed according to the plate map below. The concentration of the cells was 10^5 cells/mL with 1 mL of each cell line in each of the wells (to form a 1:1 ratio for the co-culture). Within this protocol, more controls were added- there was a plate with only macrophages and a plate with only tumor cells- in order to dictate whether any effects were happening within the diversified tumor microenvironment, or without other cells in the culture. In order to create these controls, the extra solution from the cell suspension for both the J744A.I and R1LWT cells were stored in the fridge until the samples were acquired at the flow cytometer. All the plates were then incubated for 48 hours at 37°C and 5% CO_2 to prepare for flow cytometry.

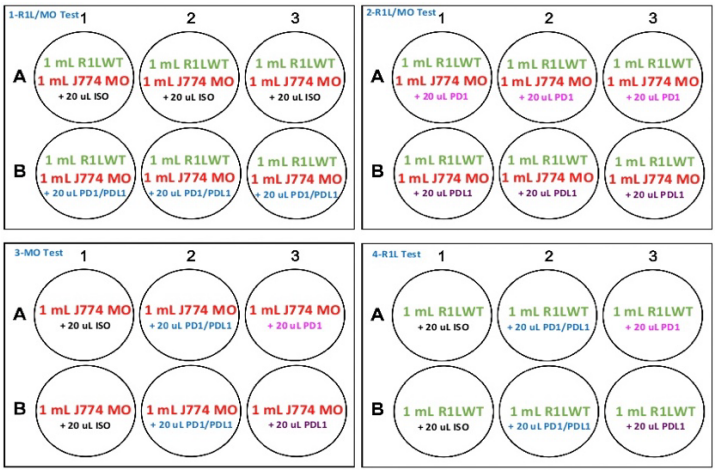

Figure 4: Plate Map for all experiments with R1LWT cells

Flow Cytometry Procedure

The flow cytometry procedure was the same as for that of Protocol 1 for B16F10 cells, except the melanoma cells were replaced with R1LWT and there was an addition of cell controls along with the single color controls (beads). Furthermore, since R1LWT didn't exhibit GFP, the expression of CTV would instead show proliferation, requiring the utilization of a different channel on the Symphony Flow Cytometer (V 431/28). Also, for beads, a different antibody was used, CD45 PB, to account for cell proliferation. All these changes are represented in the table below.

Table 2: R1LWT Antibody Panel

ANTIBODY PANEL			
CHANNEL	**MARKER**	**COLOR**	**Antibody MIX (1400 uL)**
V 431/28	Proliferation	CTV	-
YG 586/15	MHC-II	PE	7 uL
YG 610/20	Viability	ZombieRed	1.5 uL
R 670/30	MHC-I	APC	7 uL
R 780/60	CD45	APCy7	3.5 uL
SINGLE COLOR (Beads)			
CHANNEL	**MARKER**	**COLOR**	**Antibody**
Unstained	-	-	
V 431/28	Proliferation	CTV	CD45 PB
YG 586/15	MHC-II	PE	MHC-II PE
YG 610/20	Viability	ZombieRed	Ly6C PE-CF594
R 670/30	MHC-I	APC	MHC-1 APC
R 780/60	CD45	APCy7	CD45 APCy7
EXTRA CONTROLS			
CHANNEL	**TUBE**	**COLOR**	**FUNCTION**
Unstained	J774 FRIDGE	UN	FLUO. BACKGROUND

Unstained	RL1WT FRIDGE	UN	FLUO. BACKGROUND
V 431/28	RL1WT FRIDGE	CTV	MAX. CFSE COLOR

Results

B16F10-GFP-OVA Melanoma Cells

Gaiting

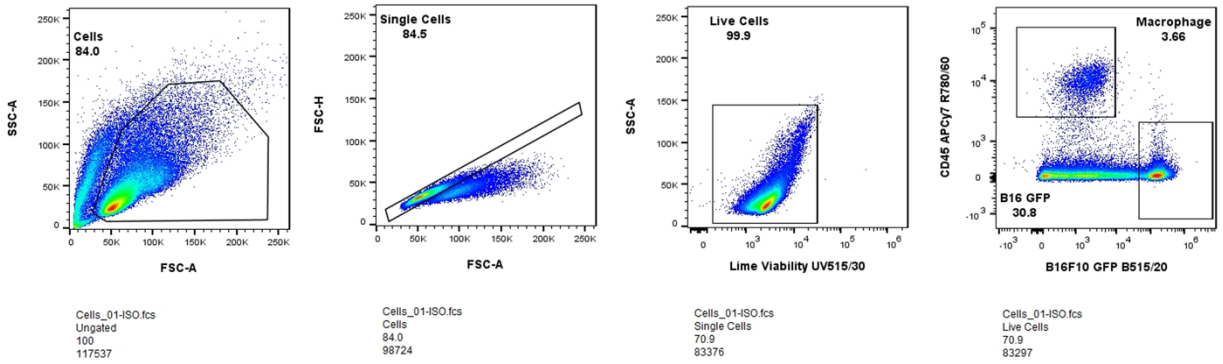

Figure 5: Gaiting protocol for B16F10 cells (divides cells into singlets, live cells, and macrophage/tumor)

Graphs

Figure 6a

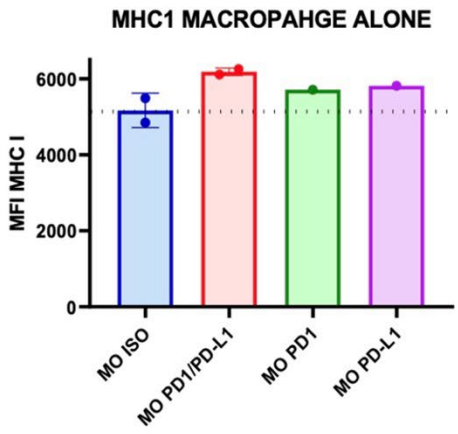

Figure 6b

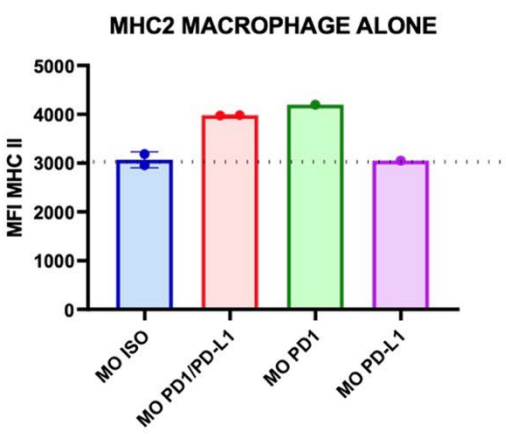

Figure 6c

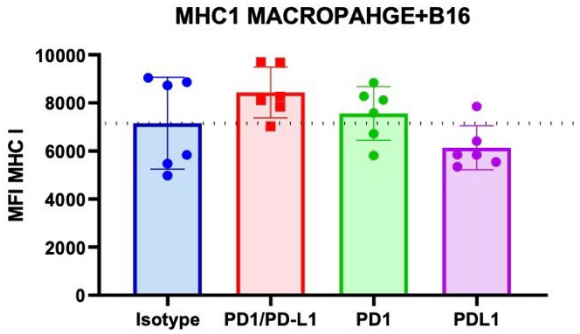

Figure 6d

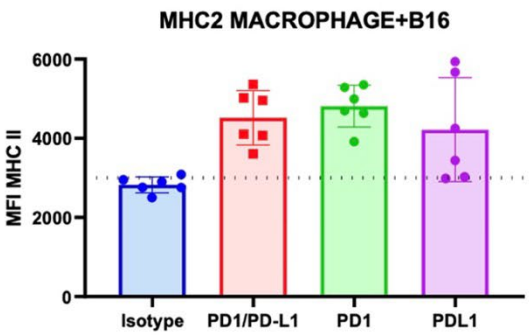

Figure 6: Both 6a and 6b show results for samples with only macrophages, where there is minimal MHCI/MHCII activation as compared to 6c and 6d, where a co-culture of tumor cells and macrophages has led to increased MHCI/MHCII activation (especially with PD1).

R1LWT Hepatocellular Carcinoma cells

Gaiting

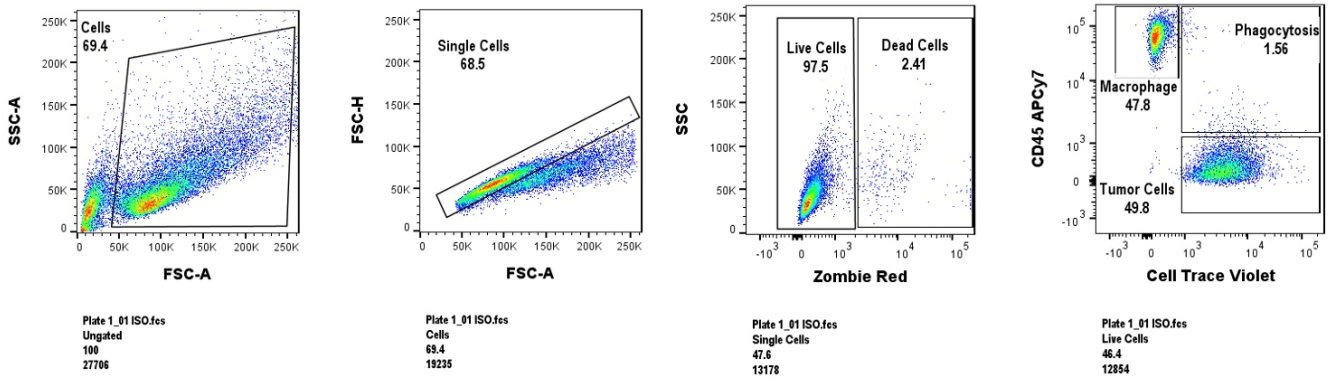

Figure 7: Gaiting protocol for R1LWT cells (separates cells into singlets, live/dead, and macrophage/tumor cells)

Graphs

Figure 8a

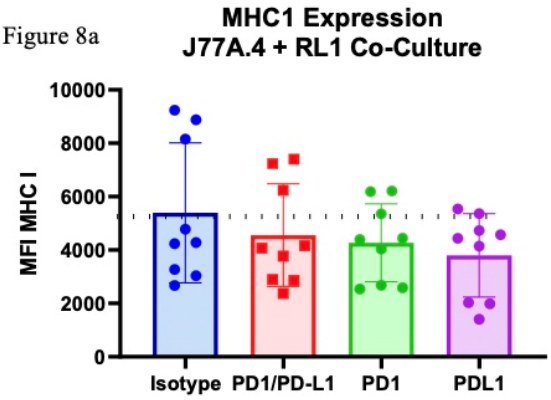

Figure 8b

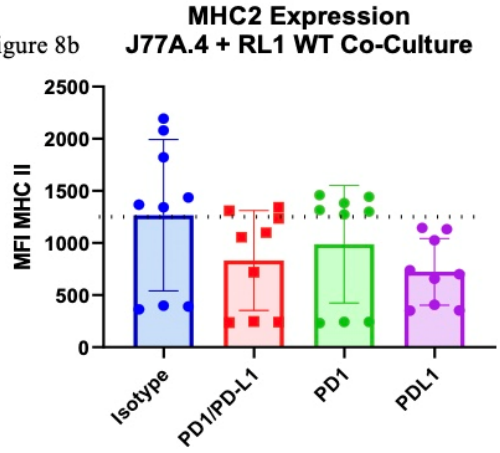

Figure 8c

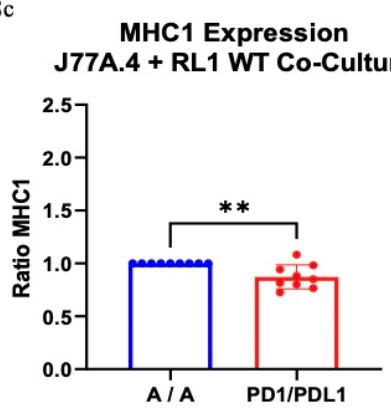

Figure 8d

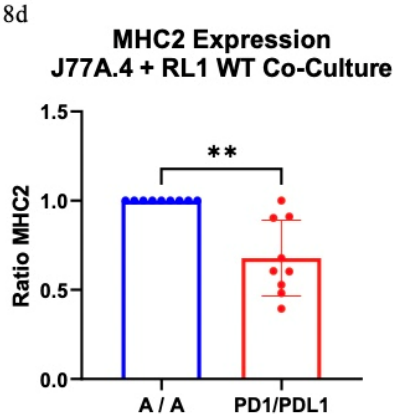

Figure 8: 8a and 8b show MHCI and MHCII expression, respectively. Figures 8c and 8d show there is no statistical significance between the MHCI/MHCII activation in a co-culture with or without antibodies.

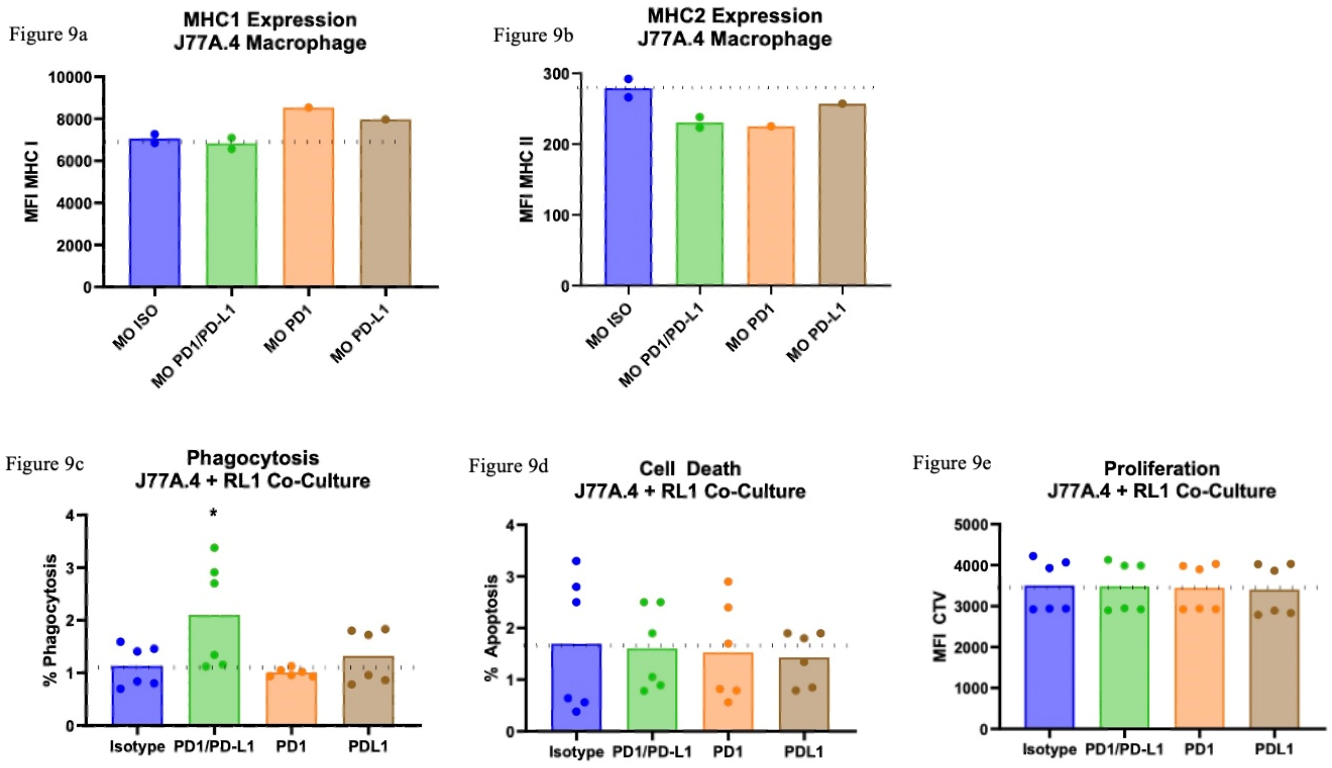

Figure 9: 9a and 9b how MHCI and MHCII activation, respectively, in samples with only macrophages. 9c shows phagocytosis levels, 9d shows cell death percentages in a co-culture, and 9e shows proliferation rates in a co-culture.

Discussion

B16F10-GFP-OVA Melanoma Cells

When treated with PD1/PD-L1 antibodies, the macrophages overall expressed more MHCI and MHCII. MHCI and MHCII are glycoproteins, with MHCI presenting endogenous antigens and MHCII presenting exogenous antigens (Kenneth 16). This increased expression allows efficient T-cell recognition, which raises antitumor activity. However, MHCII expression was greater on average (Figure 6b and 6d) when compared to MHCI (Figure 6a and 6c), likely because the B16F10 cells were extracellular cells. Combined with the fact that macrophage phagocytosis of tumor cells was low for the B16F10 cells, the higher levels of MHCII expression can be justified, since most of the activity is occurring outside of the macrophage.

Furthermore, by treating some cells with isolated PD1 or PD-L1 instead of the combination of PD1/PD-L1, the results demonstrate how the PD1 antibodies generated an antitumor effect more than the PD-L1. Anti-PD-L1 antibodies merely block overexpression of PD-L1, limiting a tumor cell's protection against cytotoxic T-cell activity. On the other hand, anti-PD1 antibodies not only suppress a tumor cell's resistance, but also enhance antigen-specific antibody responses (Hashem 2017). As the PD1 antibodies would activate beneficial immune responses, this illustrates how MHCI and MHCII expression was higher in the presence of anti-PD1 rather than PD-L1.

R1LWT Hepatocellular Carcinoma Cells

As can be seen in Figure 8a and 8b, MHCI and MHCII expression either stayed constant or decreased in the presence of PD1/PD-L1 antibodies, an effect opposite of that with the B16F10 cells. With only macrophages, as seen in Figures 9a and 9b, the MHCI/MHCII activation was similarly unchanged, as the slight increase/decrease in the activation had no statistical significance. This provides no insight as to whether macrophages only express MHC/MHCII in the presence of HCC cells, as there are no positive activation to compare this negative feedback to. Furthermore, Figures 8c and 8d prove that there is no statistical significance between the isotype and samples treated with antibodies (it is statistically significant when there are three stars above the line connecting the two bars of data), stipulating that the PD1/PD-L1 had no effect on MHCI and MHCII expression.

While the antibodies yielded no varied results in MHCI and MHCII expression, the percent phagocytosis did increase as seen in Figure 9c. This was quantified by analyzing the number of cells between the macrophage and tumor cell gaits in the graph comparing CD45 APCy7 and Cell Trace Violet in Figure 7. Essentially these were the cells that were positive for both the macrophage and tumor cell markers, demonstrating that these cells were undergoing phagocytosis. However, cell death and proliferation were unchanged in the presence of antibodies (Figures 9d and 9e), again aligning with the previous results of constant MHCI and MHCII expression for both isotype and treated samples for the R1LWT cell line.

Conclusions

When treated with PD1/PD-L1 antibodies, MHCI and MHCII activation increased in the samples with B16F10 cells, but had the opposite effect with R1LWT cells. MHCI and MHCII activation within the samples including B16F10 cells was greater with PD1 in comparison to PD-L1.

Limitations

B16F10-GFP-OVA Melanoma Cells

When running the samples through flow cytometry, the macrophage and tumor cell ratio wasn't 1:1, which could've limited the visualization of the interaction between these two cell lines. The samples often had smaller quantities of macrophages because the J774A.1 cell line would adhere onto the flask/well more powerfully than the tumor cells, requiring more vigorous resuspension. Especially since the macrophage death rate was high when using the detaching agent Trypsin (0.05% trypsin-1 mM EDTA), the later experiments within this protocol called for Versene instead. This agent wasn't as potent as Trypsin, so the macrophages didn't detach as effectively, resulting in the lower percentages of macrophages. Fortunately, when moving onto Protocol 2 with R1LWT, the macrophage and tumor cell ratio was roughly 1:1, because of a greater focus on extensive resuspension.

R1LWT Hepatocellular Carcinoma Cells

The results for samples with macrophages alone shown in Figures 9a and 9b had fewer data points than desired. These controls were only performed for one experiment out of the three conducted with the R1LWT cell line, so there was not enough data to confirm the results seen with only macrophages. Additionally, the unmodified expression of MHCI and MHCII seen in R1LWT cells could be because they don't express the PD-L1 protein as readily as the B16F10 melanoma cells. Consequently, the anti-PD1/PD-L1 antibodies would've had little to no impact on MHCI and MHCII activation. This would be a significant limitation to any future experiments that can be conducted with the R1LWT cell line.

Future Research

This study only proves that PD1/PD-L1 antibodies had no effect on MHCI and MHCII expression in R1LWT, opposite of the initial hypothesis and unlike the increased expression in previously studied B16F10 cells. In order to consider the various factors that could lend to these results, future research would have to perform multiplex assays to map out the exact pathways that result in MHCI/MHCII expression in R1LWT cells. However, multiplex assays require specific machines and tools which can be difficult to acquire for this experiment.

Thus, the next step would be to perform functional assays, which would enable studies of apoptosis/proliferation and phagocytosis. Immune system activation can be observed by analyzing a broader tumor microenvironment in-vitro, with T cells, tumor cells, and macrophages. This would allow inspection of how MHCI/MHCII activation could promote the proliferation of T cells and whether they proliferate more in the presence of macrophages. If this is the case, there would also be more phagocytosis, as the immune system model would attack the tumor cells with increased efficiency due to MHCI/MHCII activation. This methodology can be implemented for both the B16F10 and R1LWT cell lines, as varying results point towards contrasting pathways and tumor microenvironments. By performing functional assays on both cell lines, these differences can be determined, and the rates of proliferation/apoptosis can be studied in the presence of antibodies versus without.

Additionally, the role of MHC1 and MHCII in T cell proliferation and tumor apoptosis/phagocytosis can be studied by blocking the MHCI/MHCII activation in the microenvironment. If the phenomenon of anti-tumor activity subsides after negatively regulating MHCI/MHCII activation, it can be confirmed that these molecules are responsible for the results. Again, this can be performed for both B16F10 and R1LWT cells, as it can be beneficial to understand how modulating MHCI/MHCII can affect the rates of anti-tumor activity in these different cell lines. It would assist in further discovering the cellular pathways that result in tumor phagocytosis within the microenvironment.

Finally, the cell lines could be studied extensively in-vivo, as a mouse's tumor microenvironment has other immune cells that might affect MHCI/MHCII activation. It would also provide results on whether MHCI/MHCII activation would induce T-cell proliferation and ultimately kill tumor cells. In previous studies, anti-PD-L1 antibodies have helped reduce tumor weight and tumor volumes with B16F10 cells in-vivo, but only slightly (Zhengping 2018). It would be interesting to analyze these results with both cell lines so that pathways can be further analyzed. Because of the previous speculation regarding low levels of PD-L1 protein in R1LWT cells, it would be intriguing to discover whether anti-PD1/PD-L1 antibodies could target other immunosuppressive pathways, such as cytokine and metabolic pathways. Such a study would be more comparable to human studies as well, since increased tumor cell apoptosis in-vivo would be more indicative of patient responses to these antibodies.

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