Investigation of pancreatic tumor cell intrinsic differences in capacity to activate tumor-specific T cells

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12/11/18

Abstract

Pancreatic ductal adenocarcinoma (PDA), is the most common form of pancreatic cancer which can be modeled in mice. There are two district morphological types of PDA cells: cuboidal and mesenchymal. In this line of experimentation, it has been found that both cell types do not activate T cells from endogenous antigen sources, however, when a peptide is presented to these cells they can activate T cells. Based on this information future experiments need to be conducted, including determining if peptide processing machinery is present in PDA cells. With this information novel immunotherapy techniques can be developed to destroy PDA cells and eliminate the cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is an incredibly lethal form of pancreatic cancer. PDA has high mortality rates for three main reasons: it is highly metastatic, most patients are diagnosed at advanced stages, and current treatments are not effective (Stromnes et al., 2014). Pancreatic cancer is able to evade cancer immunotherapy, a promising therapeutic technique. Cancer immunotherapy is designed to stimulate a patients' immune system, allowing or engineering the patient's immune cells to identify and destroy the cancer cells. It is crucial to understand the mechanism by which PDA evades cancer immunotherapy as the incidence rates of pancreatic cancer are expected to rise. As recently as 2017 the incidence rate of PDA in North America was 7.2 per 100,000 people which is astonishing as the incidence rate in Asia is only 2.8 per 100,000 people (Simoes et al., 2017). Additionally, in 2016, it was predicted that deaths due to PDA would exceed those from breast cancer which is

almost 5 times more common (Kenner et al., 2016). Based on this information it is predicted that by 2030 PDA will become the second leading cause of cancer-related deaths, with only an 8% chance of surviving more than 5 years (Kenner et al., 2016).

Cancer immunotherapy exploits the mechanism by which the adaptive immune system protects our bodies from pathogens and cancer. The adaptive immune system contains T cells which express variable antigen-specific receptors that provide specificity and recognition, referred to as T cell receptors (TCRs) (Melvold and Sticca, 2007). T cell recognition is due to TCRs that bind peptides complexed with MHC molecules on the surface of antigen-presenting cells or cancer cells (Melvold and Sticca, 2007). This allows the T cells to find infected cells which present peptides, such as genes associated with viruses or bacteria, with MHC class I for CD8+ T cells and MHC class II for CD4+ helper T cells (Melvold and Sticca, 2007).

In 2015, Dr. Stromnes engineered T cells so that they are now able to recognize and kill pancreatic cancer cells which develop in a mouse model of PDA (Stromnes et al., 2015). Unfortunately, the tumor cells can circumvent the engineered T cells. Therefore, a greater understanding of how tumor cells can evade T cells could help develop more effective immunotherapies.

Recently, the Stromnes lab has discovered that tumor cells which arise in the PDA mouse model have two morphological phenotypes: cuboidal and mesenchymal. Therefore, I have chosen to test the hypothesis that the phenotypic differences between these cancer cells may impact T cell-mediated recognition of peptide:MHC complexes. Cuboidal cells are found within the linings of organs such as the pancreas that are involved in secretion, these cells are generally the cells which become cancerous

(Bowen; Adenocarcinoma of the pancreas, 2002). Mesenchymal cells, on the other hand, are believed to have heightened metastatic potential and invade other organs spreading the cancer (Yang and Weinberg, 2008). Previous research has concluded that genes found in mesenchymal cells are often correlated with wound healing and angiogenesis genes which have found to be associated with resistance to immunotherapy (Hugo et al., 2017). Therefore, our hypothesis is that T cells will bind and destroy cuboidal tumor cells more efficiently than they kill mesenchymal tumor cells.

Methods – gp33

First, I generated a gene block containing a model antigen (gp33) attached to green fluorescence protein (GFP) and inserted this gene into retroviral vectors using standard cloning techniques. Next, this gene block was transduced into cuboidal and mesenchymal primary tumor cells. This is required because other target antigens, such as mesothelin (Moon et al., 2011), may differ in expression between cuboidal and mesenchymal cell lines. Gp33 is a viral protein which forms the viral capsid and is thus a target for the immune system. There are two forms of gp33 that are routinely used, we have chosen to use the version that induces a stronger T cell response (Puglielli et al., 2001).

The first experimentation was to determine if the transduced cell line can process and present endogenously encoded antigen (gp33) and activate T cells. As a negative control, we used the parental tumor cell lines. As a positive control, we pulsed the parental cell line and the transduced cell line with the gp33 peptide. Additionally, interferon gamma (IFNγ) was added to half of the cells because IFNγ is a cytokine that

is crucial for immunity against viral infections, and for MHC class I upregulation (Ikeda et al., 2002). In order to test this, each of the cell lines (transduced cuboidal, and parental cuboidal) were plated in 8 wells of a 96 well plate at a concentration of $1x10^4$ cells per well. The peptide and IFNγ were added to the appropriate wells each as a 1:1000 dilution. Then, a naïve spleen from a P14 TCR transgenic mouse that expresses TCRs specific to gp33/H-2Db MHC was harvested. The spleen was then pulverized to expose the T-cells. $1x10^6$ spleen cells were then be added to each well and the cells were returned to the 36 degree, 5% CO2 incubator. After 24 hours, the cells were re-suspended in media and spun in the centrifuge. The cells were then re-suspended in FACs buffer with antibodies for CD8 T cells (CD8) and T cell activation (CD69, CD25). Finally, the cells were analyzed with flow cytometry.

Methods – Ova

As seen in the data section the transduction of the mesenchymal cell line with the gp33 gene block was not successful. In order to test how mesenchymal cells activate T cells two cell lines that were previously transduced with ovalbumin were used. Ovalbumin (ova) is a protein found in the whites of chick eggs and therefore a target for the mouse immune system (Lv et al., 2015). Cells from these two cell lines (mesenchymal 1 and mesenchymal 2) were previously implanted into mice and tumor cells formed. These cells were harvested and cell lines were created (re-derived mesenchymal 1 and re-derived mesenchymal 2). A similar protocol was followed with the exception of four cell lines instead of two and using an ova peptide instead of gp33.

Results and Discussion

Based on the data we can tentatively conclude that PDA cells, both cuboidal and mesenchymal, and unable to process and or present endogenously encoded peptide. Figure 1 depicts the gp33 and GFP gene block that was created. This gene block was then transduced into cuboidal and mesenchymal cell lines using an effectine kit. Figure 2 shows flow cytometric gating strategy and the results from this experiment. The cells were gated first on single cells, the next gate was live cells, and finally, GFP expression was quantified. GFP expression was used because if the cells are GFP positive then they were successfully transduced with the gene block that contains both GFP and gp33. The cuboidal cell line was transduced with 11.4% efficiency and the mesenchymal cell line was transduced with 6.18% efficiency. We generally see efficiencies above 50%, because of the minimal efficiencies obtained we decided to only proceed with the cuboidal cell line. The cuboidal cells that express GPF and gp33 were bulk sorted to create a pure population.
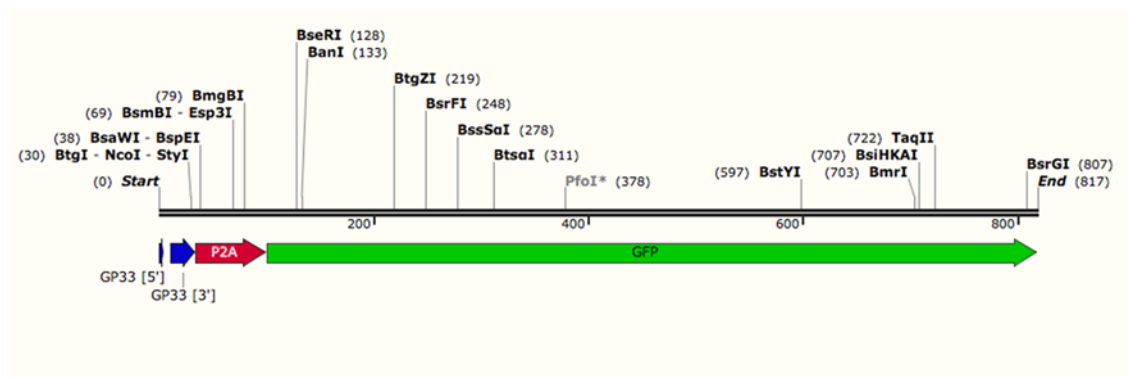

Figure 1: Gene block containing gp33 peptide linked to GFP for transducing tumor cell lines.
The gp33 epitope from Puglielli et al. was linked with a P2A to GFP to analyze transduction efficiency in tumor cells. The restriction enzyme sites along the gene block are also pictured. These sites were used to insert the gene block into a mouse retroviral vector to transduce cancer cells.
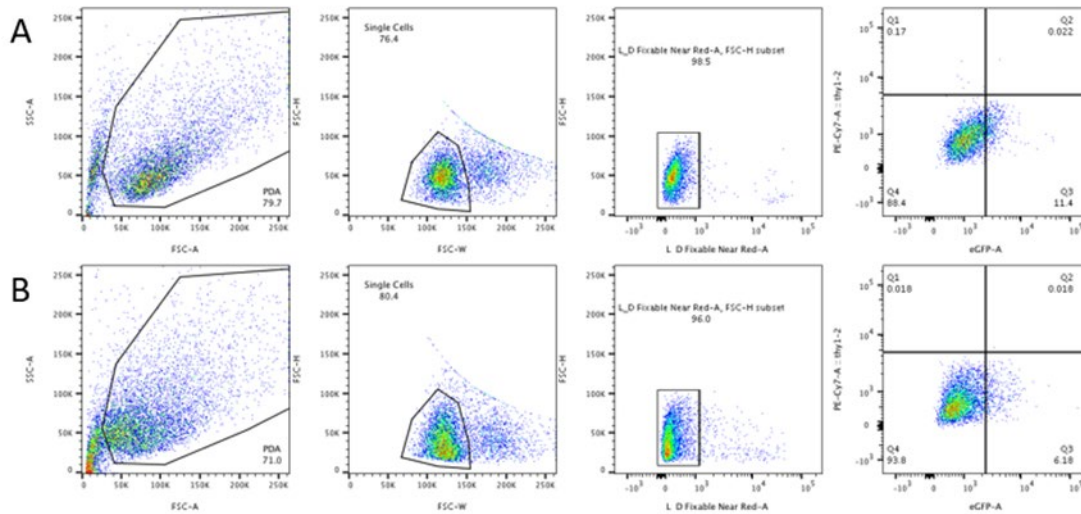
Figure 2: Flow cytometric analysis of gp33-GFP transduced cuboidal (A) and mesenchymal (B) KPC pancreatic tumor cell lines. The retroviral vector that expresses gp33 was transduced into the cuboidal and mesenchymal cell lines using an effectene transduction kit. After 3 days, the cells were analyzed for GFP expression by flow cytometry. The cells were gated on forward and side scatter which is a measure of cell size and granularity, doublets were excluded, dead cells were excluded and then GFP+ cell frequency was determined. The cuboidal cell line (A) was transduced with 11.4% efficiency, and the mesenchymal cell line (B) was transduced with 6.18% efficiency. The cell lines were sorted for GFP (data is not shown).

Figures 3 through 5 depict the results of the cuboidal gp33 T cell activation experiment. This experiment was designed to determine if PDA cells can process and present endogenously encoded neoantigen as well as an exogenously presented peptide. Figure 3 represents the flow cytometric gating strategy used to analyze the cells. The cells were first gated on live single cells, then CD8 positive T cells, and then activation was determined. Fully activated T cells are thought to express both CD25 and CD69 because these are expressed following TCR signaling. Figure 4 shows the percentage of CD8 positive T cells in each of the samples. The transduced cuboidal cell line had the largest percentage of T cells that were CD8 positive, and the parental cuboidal cell line with the gp33 peptide added had the smallest percentage of CD8

positive T cells. Based on figure 4 we can conclude that the presence or absence of gp33 (endogenous or exogenous) and the presence or absence of IFNγ does not impact the percentage of T cells that are CD8 positive. Figure 5 shows the percentage of the CD8 positive T cells in each sample that are fully activated. Based on this data, endogenous gp33 was not sufficient to fully activate the CD8 T cells like the exogenous gp33 peptide. Therefore we can conclude that cuboidal PDA cells are not able to process and present endogenously encoded antigens and fully activate T cells.
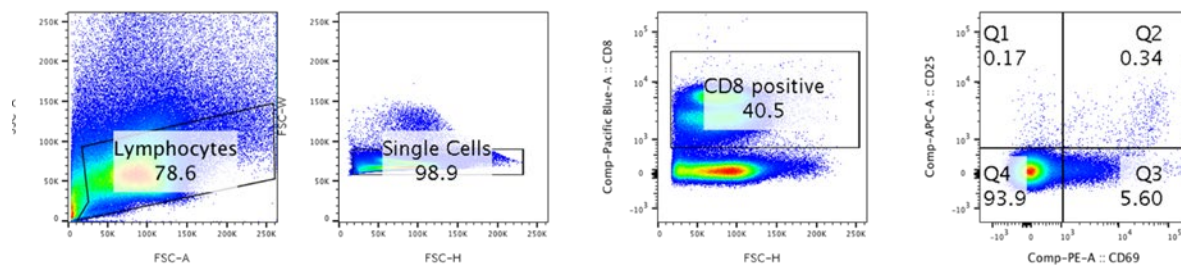

Figure 3: Flow cytometric analysis of CD8+ T cell activation 24 hours after incubation with the gp33-transduced cuboidal cell line. T cells were harvested from a naïve p14 mouse spleen. These cells were then incubated in combinations ± gp33 peptide and IFNγ. The peptide was used as a positive control and the IFNγ was used to increase MHC class I binding activity. This figure depicts the gating strategy used to determine if the T cells were fully activated (CD25 and CD69 positive).
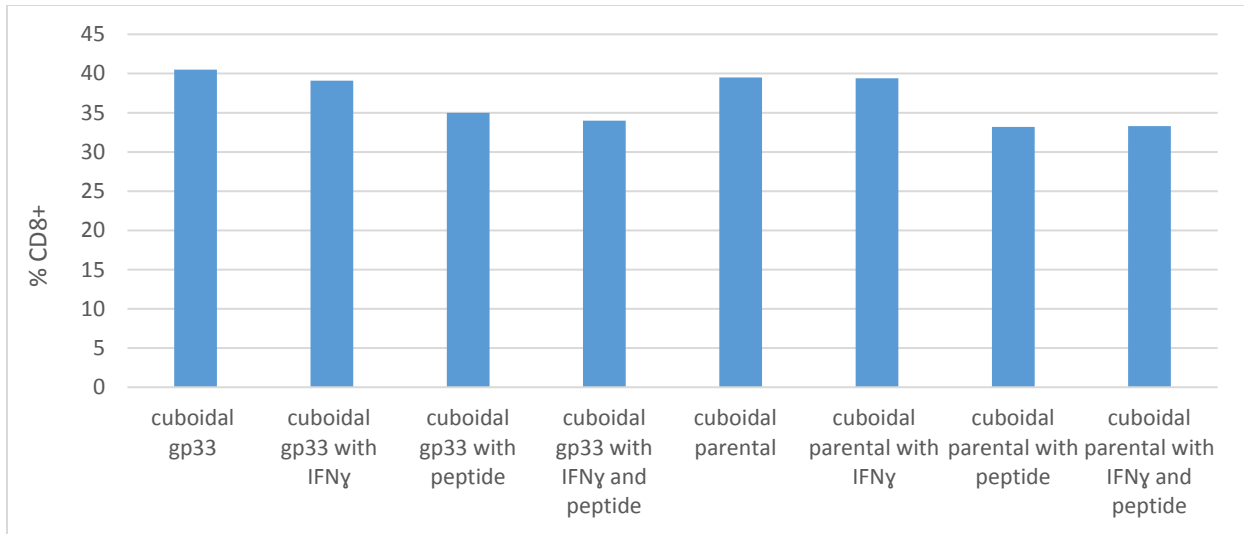
Figure 4: The percentage of CD8+ T cells after incubation with the cuboidal tumor cells that expressed or did not express gp33 with the indicated conditions.
The cuboidal cells were transduced with a gp33/GFP vector and the parental cells are untransduced. These cells were incubated in combinations ± gp33 peptide and IFNγ. The peptide was used as a positive control and the IFNγ was used to increase MHC class I binding activity. The percentage of T cells that were CD8 positive was determined using flow cytometric analysis.
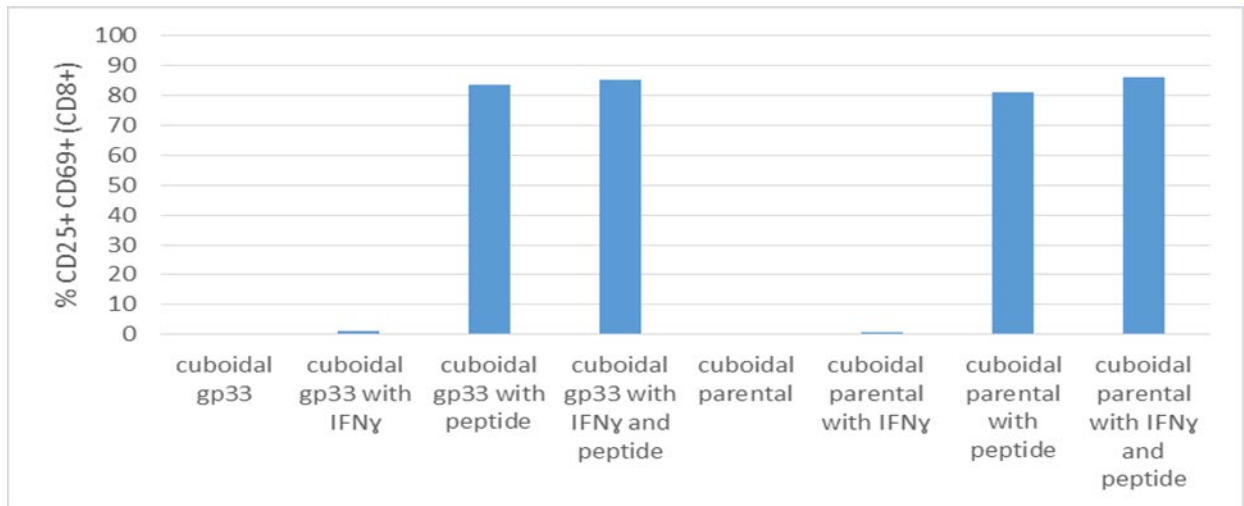

Figure 5: The percentage of CD8+ T cells that are CD69 and CD25 positive after incubation with cuboidal tumor cells that did or did not express gp33 with the indicated conditions.
CD69 and CD25 are markers of T cell activation. CD8 T cells that express CD69 and CD25 are considered fully active. T cells were incubated with parental or transduced cuboidal tumor cells ± gp33 and IFNγ. The activation of the CD8+ T cells was determined with flow cytometric analysis.

Figures 6 through 8 depict the results of the mesenchymal ova T cell activation experiment. This experiment was designed to determine if PDA cells can process and

present endogenously encoded neoantigen as well as an exogenously presented peptide. Figure 6 represents the flow cytometric gating strategy used to analyze the cells. The cells were first determined to be single cells, then CD8 positive T cells, and then activation was determined. Fully activated T cells are thought to express both CD25 and CD69. Figure 7 shows the percentage of CD8 positive T cells in each of the samples. The mesenchymal cell line 1 without ova peptide or IFNγ had the largest percentage of T cells that were CD8 positive, and the re-derived mesenchymal cell line 2 with the ova peptide added had the smallest percentage of CD8 positive T cells. Based on figure 4 we can conclude that the presence or absence of ova (endogenous or exogenous) and the presence or absence of IFNγ does not impact the percentage of T cells that are CD8 positive. It is notable that the percentage of CD8 T cells is very different between the cuboidal and mesenchymal cell lines as seen by comparing figures 4 and 7. This difference will need to be further validated with future experiments. Figure 8 shows the percentage of the CD8 positive T cells in each sample that are fully activated. Based on this data it is obvious that the endogenous ova was not sufficient to fully activate the CD8 T cells like the exogenous ova peptide. Therefore we can conclude that cuboidal PDA cells are not able to process and present endogenously encoded antigens and fully activate T cells. From this experiment, we can also conclude that the re-derivation process, placing the cell line in a mouse, harvesting the tumor that forms, and creating a cell line, does not alter a cell lines ability to present exogenous presented peptide. This allows us to conclude that the re-derived cells did not lose MHC-class I expression, however, we do not have the data to confirm that they did not lose their endogenous ova peptide.
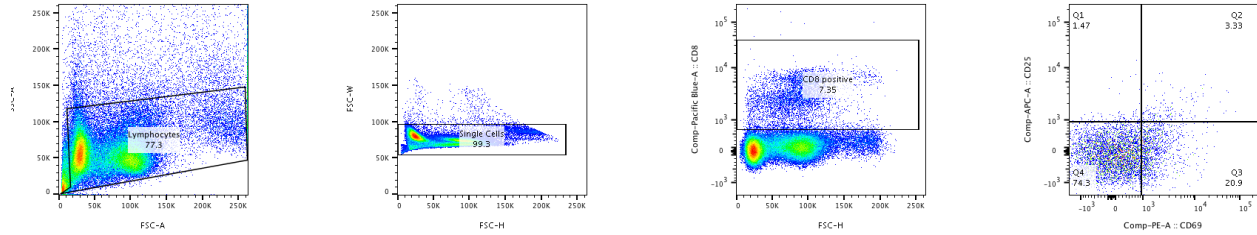
Figure 6: Flow cytometric analysis of CD8+ T cell activation 24 hours after incubation with the ova transduced mesenchymal line

T cells were harvested from a naïve ot1 mouse spleen. These cells were then incubated in combinations ± ova peptide and IFNɣ. The peptide was used as a positive control and the IFNɣ was used to increase MHC class I binding activity. This figure depicts the gating strategy used to determine if the T cells were fully activated (CD25 and CD69 positive).
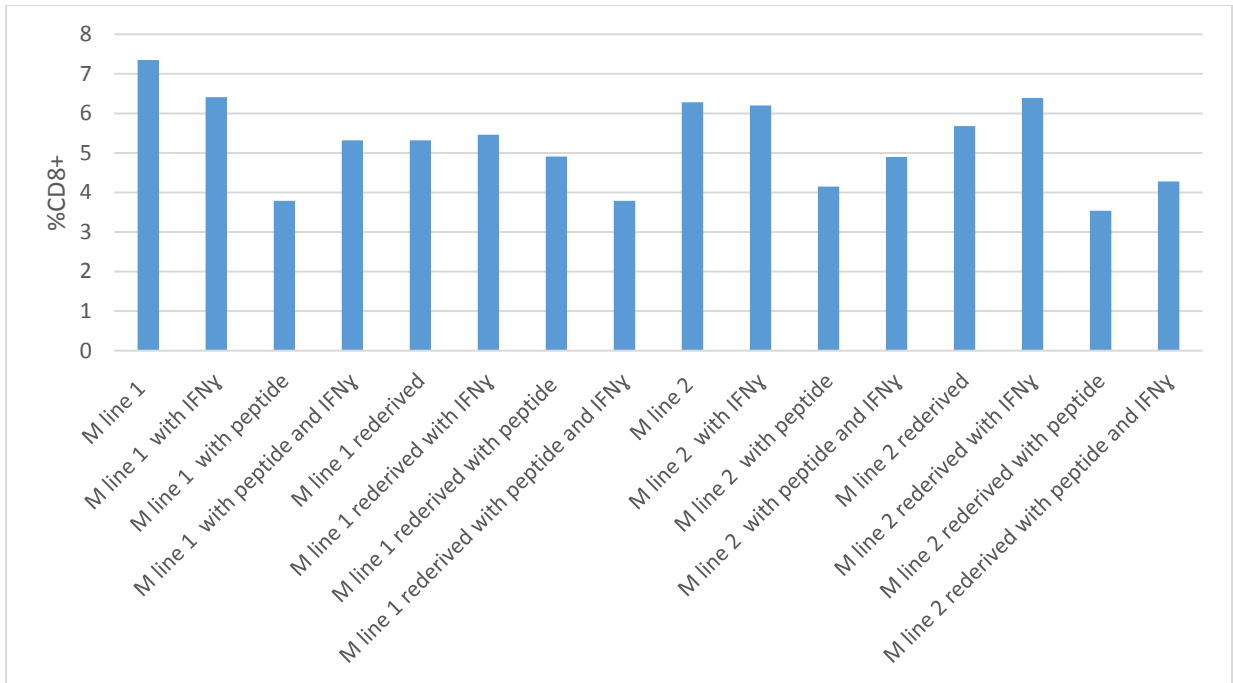
Figure 7: The percentage of CD8 + T cells after incubation with mesenchymal tumor cells that express ova with the indicated conditions.
The mesenchymal cells were transduced with an ova vector. These cells were then injected into mice to create a new tumor. This tumor was harvested and the rederived cell lines were created. These cells, transduced and rederived, were incubated in combinations ± ova peptide and IFNɣ. The peptide was used as a positive control and the IFNɣ was used to increase MHC class I binding activity. The percentage of T cells that were CD8 positive was determined using flow cytometric analysis.
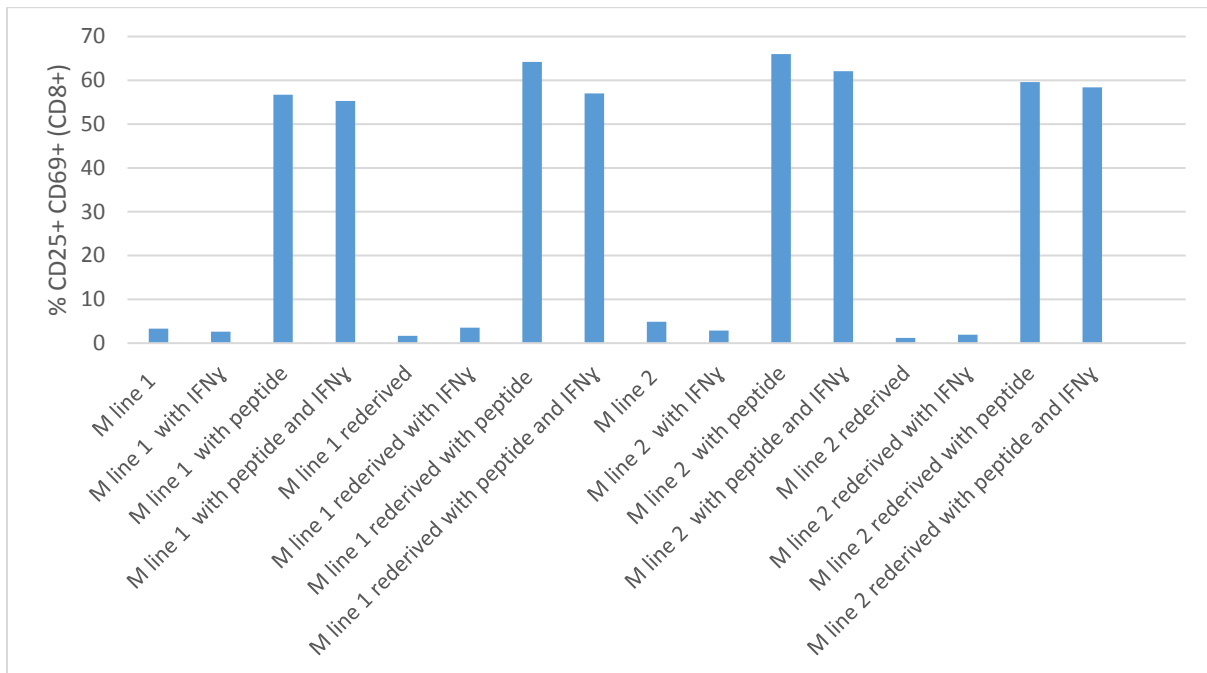
Figure 8: The percentage of CD8+ T cells that are CD69 and CD25 positive after incubation with mesenchymal tumor cells that express ova with the indicated conditions.
CD69 and CD25 are markers of T cell activation. CD8 T cells that are both CD69 and CD25 positive are considered fully active. T cells were incubated with transduced and rederived mesenchymal tumor cells ± ova and IFNγ. The activation of the CD8+ T cells was determined with flow cytometric analysis.

Future Directions

The data presented leads to more questions than answers. Therefore, future experiments need to be conducted. These future experiments will confirm the presence of the model antigens, confirm if there are deleterious effects on the CD8 T cells by incubating with the tumor cell lines, and compare the PDA cell lines to melanoma cell lines, which are known to be highly immunogenic, and investigate the presence of antigen processing machinery.

The first confirmation that needs to be conducted is to confirm the presence of the model antigens in the cuboidal and mesenchymal PDA cell lines. This can be qualitatively confirmed with a fluorescent microscope. This is because in the cell lines the model antigens gp33, and ova, are attached to GFP which can be visualized. The presence of the model antigens can also be quantifiably confirmed via flow cytometric sorting based on GFP. This data will confirm that our cells have the endogenous antigen and are not able to process and present it like they do with the exogenous peptide.

Secondly, the cuboidal and mesenchymal PDA T cell activations need to be repeated. This is because the percentage of CD8 positive T cells varies fairly drastically between the cell lines as seen in figures 4 and 7. This data will confirm or refute this

variation. If the cell lines produce different amounts of CD8 T cells further experimentation will be needed to understand why. For example, it is possible that the mesenchymal cell lines produce factors that are inhibitory to T cells.

The third experiment to be conducted will be to replicate the T cell activation experiments with a melanoma cell line that has been transduced with ova. There are currently multiple immunotherapy drugs that have been approved to treat melanoma including ipilimumab and nivolumab (Albertini, 2018). Therefore, our hypothesis is that the ova melanoma cell line is more likely to be able to process and present endogenous antigens. If this is true then we can conclude that there is something wrong with PDA cells specifically that does not allow the processing and presentation of endogenous antigens.

We know the PDA cells contain MHC class I which allows for antigen presentation because they are able to present the exogenous peptide and activate CD8 T cells. Additionally, based on the fact that we can re-derive tumors from these cell lines we can conclude that these cells, even with the modification, can escape the immune system. Therefore, we posit that PDA cells are not able to process the endogenous antigens and this is a critical immune evasion strategy. Antigens are processed into peptides which are then recognized by the MHC class I complexes (Blum et al., 2013). The processing is accomplished by the proteasome, which chops up the protein into peptide and this process is induced by IFNγ (Blum et al., 2013). In order to determine if the proteasome is present in these PDA cells and how active it is we plan to use western blots, which show what proteins are present. I will use a western blot to determine the presence of three proteins TAP, LMP2, and LMP7. The TAP transporter is a pump that

transports peptides to the endoplasmic reticulum from the cytoplasm (Murata et al.,

2018). LMP2 and LMP7 are immunoproteasome subunits that have peptidase activity

(Murata et al., 2018). If these proteins are found in the PDA cell lines similar to a control

melanoma cell line, then the proteasome is functional and other theories will need to be

explored.

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