Using a leptin containing oncolytic vaccinia virus to reprogram the tumor microenvironment to increase T cell function

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The tumor microenviroment can restrict the metabolic activity of T cells, preventing their effector function, proliferation, and tumor clearance. Altering the metabolic landscape towards the benefit of the T cells has been shown to reverse these effects. The adipokine leptin is produced by adipocytes in proportion to the body's energy stores. As receptors are expressed across immune cell types the levels of leptin in the body can have a profound effect on immune function. Higher leptin is correlated to higher effector function in T cells. The delivery of leptin to a tumor using an oncolytic vaccinia virus (leptin-vv) may increase the proliferative ability and decrease exhaustion of T cells while decreasing tumor growth. These effects to tumor growth are limited to intratumorally injected leptin-vv while the immune changes are seen in both intratumoral and intravenous deliveries.

INTRODUCTION

The tumor microenvironment (TME) restricts the function of T cells in a variety of ways including recruitment of suppressive cell types (regulatory T cells, MDSCs), use of checkpoint inhibitors (PD-L1), as well as creating a metabolically deficient environment. As tumor cells are constantly dividing they have high consumption of glucose, amino acids, and oxygen which restricts the access of immune cells such as infiltrating T cells to access them. This effects the T cells ability to infiltrate, proliferate, and perform their effector functions(1,2). Targeting the metabolic side of the tumor's suppressive ability to improve T cell function is being explored by the Delgoffe lab and others. Previous work in the Delgoffe lab has shown some success in this field. They have shown that decreasing the oxygen consumption of the tumor itself can increase sensitivity to immunotherapy (αPD1) and increase the T cell tumor response(3). It has also been shown that tumor infiltrating T cells have decreased mitochondrial mass and function but enforcing mitochondrial biogenesis can improve their effector function(1).

As a result of the growing data to support the metabolic suppression of T cells the adipocyte produced hormone leptin was tested for its ability to modulate T cell effector function. Leptin is an adipokine produced by adipocytes in proportion to triglyceride stores in the body and as such is a reflection of the bodies current energy stores(4).The circulating concentration of leptin can become altered with nutritional dysfunction; leptin is high in cases of obesity and low in cases of malnutrition(5). Importantly leptin receptor (lepR) is expressed on the surface of many immune cells across both innate and adaptive immunity and can affect the immune response(5). It has been shown in fasting mice with low leptin levels that T cell activation and glucose uptake are defective(5). This effect on glucose uptake however was not shown to extend to regulatory T cells and in fact leptin has been shown to decrease the proliferation of T-regs(6).

Previous work in the Delgoffe lab has demonstrated the ability of leptin treatment *in vitro* to increase the oxygen consumption rate of T cells. When give intratumorally to PTEN-BRAF

melanoma bearing mice, leptin treatment increased the number of proliferating infiltrating T cells as well as slowed tumor growth. By tracking phosphorylation of lepR signaling molecules it was found that this treatment mechanism was triggering lepR signaling in T cells. This showed promise as a method of enhancing T cell effector function and response in the TME. It was determined that the best way to deliver the leptin to the TME would be through oncolytic virus which has the added benefit of acting as a combination treatment.

An oncolytic virus is a virus that selectively replicates only in tumor cells, leaving healthy cells intact. For this purpose, a thymidine kinase deleted (TK-) vaccinia strain was chosen. Thymidine kinase is an essential enzyme in the pyrimidine synthesis pathway. Deleting the viral TK gene results in restricting the viruses ability to replicate in only cells with high pyrimidine pools, such as rapidly proliferating tumor cells(7). Oncolytic viruses can be modified to include genes of interest; in this case the virus would also produce leptin. Alongside the benefit of directly delivering leptin to the TME, oncolytic viruses lead to immunogenic cell death(8). This mechanism leads to the release of damage associated molecular patterns which in turn activate the innate immune system to recruit the adaptive immune response. The vaccinia oncolytic virus specifically triggers immunogenic apoptosis, necrosis, and autophagic cell death leading to activation of macrophages and dendritic cells, recruitment of neutrophils, the production of IFNγ and TNFα, and antigen-specific activation of CD8+ T cells(8).

We believe the combination of the anti-tumor effects produced by the oncolytic virus with the metabolic reprogramming effect of leptin will increase T cell recruitment to the tumor and effector activity. While work has been done in the lab testing leptin and the leptin-vv as a treatment with intratumoral injection this method would not work for many tumor types where the tumor is not easily accessible. It became crucial to elucidate if any anti-tumor effects were visible with an intravenous injection.

MATERIALS AND METHODS

Tumor Growth and Treatment

Mice were injected with 250,000 PTEN-BRAF Clone 24 cells subdermally. Clone 24 is a cell line that was generated from a transgenic PTEN-BRAF tumor. After injection tumor growth was monitored and on day 8 treatment was administered. Mice were treated with either 50 µL of PBS intravenously, 50 µL of 10^6 PFU of leptin-vv intratumorally (IT), or 50 µL of 10^8 PFU of leptin-vv intravenously (IV, 2 mice per group). Mice were imaged on the IVIS one day post-treatment. Mice were given 100 µL of luciferase reagent injected intraperitoneally 5 minutes before imaging. One hour prior to harvesting tumors and lymph nodes 100 µL of hypoxyprobe reagent was injected intravenously to all mice.

Tissue Processing

Tumors and lymph nodes were harvested into complete RPMI media. Lymph nodes were mechanically disassociated and run through a 70 µm filter. Tumors were incubated for 20 minutes at 37˚C in an enzyme cocktail containing dispase, DNase, and collagenase in serum-free RPMI followed by mechanical dissociation and filtration through a 70 µm filter. Tumor samples were

then incubated at room temperature for 1 minute in red blood cell lysis and quenched with media. Lymph node and tumor cells were aliquoted for staining.

Overnight Stimulation

One set of lymph node and tumor cell samples were plated for overnight stimulation (Cytokines, Table 1). Half of the samples were give complete RPMI media containing PMA and ionomycin (stimulated) while half were given complete media only (unstimulated). These cells were incubated overnight at 37˚C. The next morning golgi plug was added and cells were incubated at 37˚C for 5 hours prior until staining.

Live Staining for Flow Cytometry

One panel was run on live cells (Metaboexhaustion, Table 1). These cells were incubated at 37˚C for 20 minutes in RPMI containing 2NBDG to measure their glucose uptake. After incubation cells were washed in flow buffer (PBS, 2% FBS) and stained with surface markers for 15 minutes on ice. After another flow buffer wash secondary streptavidin was added for 10 minutes on ice. Cells were again washed, resuspended, and samples were run on the LSR Fortessa at the Hillman Cancer Center flow core.

Fixed Staining for Flow Cytometry

Surface staining was carried out in the same manner as above. After streptavidin staining, cells were incubated in the Fix and Perm Cell Permeabilization kit (Invitrogen) for 20 minutes at room temperature. After the fix/perm step cells were washed with the fix/perm buffer supplied with the kit. Cells were incubated in the intracellular antibody cocktail until samples were run at the Hillman Cancer Center flow core. On the day of the run cells were again washed with fix/perm buffer, resuspended in fix/perm buffer and then analyzed on the LSR Fortessa.

LSRFortessa	Cytokine/Txn (overnight stim)		
Fluorochrome	Filter	Antibody	Dilution
FITC/GFP/YFP	488_515_20	CD8a	1000
PerCP-Cy5.5	488_710_50	*TNF*	500
PE, MitoOrange	561_582_15	*Ki67*	250
PE-TR, 594, RFP	561_610_20	*T-bet*	200
PE-Cy5.5	561_710_50		
PE-Cy7	561_780_40	*IL-2*	200
Pac Blue/BV421	405_450_50	Hypoxyprobe	
BV510/Ametrine	405_515_20	*IFNg*	250
BV605	405_660_40		
BV711	405_710_40		
BV786	405_780_40	PD-1	500
APC/AxF647/AxF660	628_660_50	*Eomes*	200
AxF700	628_730_45		
APC-Cy7	628_780_60	CD4	500

LSRFortessa	Myeloid populations		
Fluorochrome	Filter	Antibody	Dilution
FITC/GFP/YFP	488_515_20	VDAC	500
PerCP-Cy5.5	488_710_50		
PE, MitoOrange	561_582_15	hypoxyprobe	1000
PE-TR, 594, RFP	561_610_20		
PE-Cy5.5	561_710_50	Ly-6C PE Cy7	500
PE-Cy7	561_780_40	TCR β chain - PE Cy5	250
Pac Blue/BV421	405_450_50	CD11b - Pac Blue	250
BV510/Ametrine	405_515_20	CD11c - BV510	250
BV605	405_660_40	F4/80 - BV650	250
BV711	405_710_40		
BV786	405_780_40		
APC/AxF647/AxF660	628_660_50	Mitotracker	1000
AxF700	628_730_45		
APC-Cy7	628_780_60	leptin R (biotin)	250

LSRFortessa	T-NK activation		
Fluorochrome	Filter	Antibody	Dilution
FITC/GFP/YFP	488_515_20	VDAC	500
PerCP-Cy5.5	488_710_50	B220	250
PE, MitoOrange	561_582_15	Hypoxyprobe	
PE-TR, 594, RFP	561_610_20		
PE-Cy5.5	561_710_50		
PE-Cy7	561_780_40	CD8	1000
Pac Blue/BV421	405_450_50	CD44	500
BV510/Ametrine	405_515_20		
BV605	405_660_40	Ki67 BV605	500
BV711	405_710_40		
BV786	405_780_40	CD4	500
APC/AxF647/AxF660	628_660_50	NK1.1	200
AxF700	628_730_45	Foxp3	200
APC-Cy7	628_780_60	Leptin	100

LSRFortessa	Metaboexhaustion		
Fluorochrome	Filter	Antibody	Dilution
FITC/GFP/YFP	488_515_20	2NBDG	pulse
PerCP-Cy5.5	488_710_50	CD4 PerCP Cy5.5	500
PE, MitoOrange	561_582_15	Tim-3	250
PE-TR, 594, RFP	561_610_20		
PE-Cy5.5	561_710_50		
PE-Cy7	561_780_40	CD8a	1000
Pac Blue/BV421	405_450_50	Hypoxyprobe	1000
BV510/Ametrine	405_515_20		
BV605	405_660_40		
BV711	405_710_40	LAG3 BV711	250
BV786	405_780_40	PD-1 BV786	500
APC/AxF647/AxF660	628_660_50	Mito Deep Red	1000
AxF700	628_730_45		
APC-Cy7	628_780_60	Leptin	100

Table 1. **Flow cytometry panels**

RESULTS

Effect of Leptin-vv Treatment on Tumor Growth

Only treatment with leptin-vv IT slowed tumor growth (Figure 1b). The presence of the virus was only observed in the leptin-vv IT treated animals. The leptin-vv IV treated animals had no visible luminescence from the virus suggesting that virus did not reach the tumor at this time point (Figure 1c).

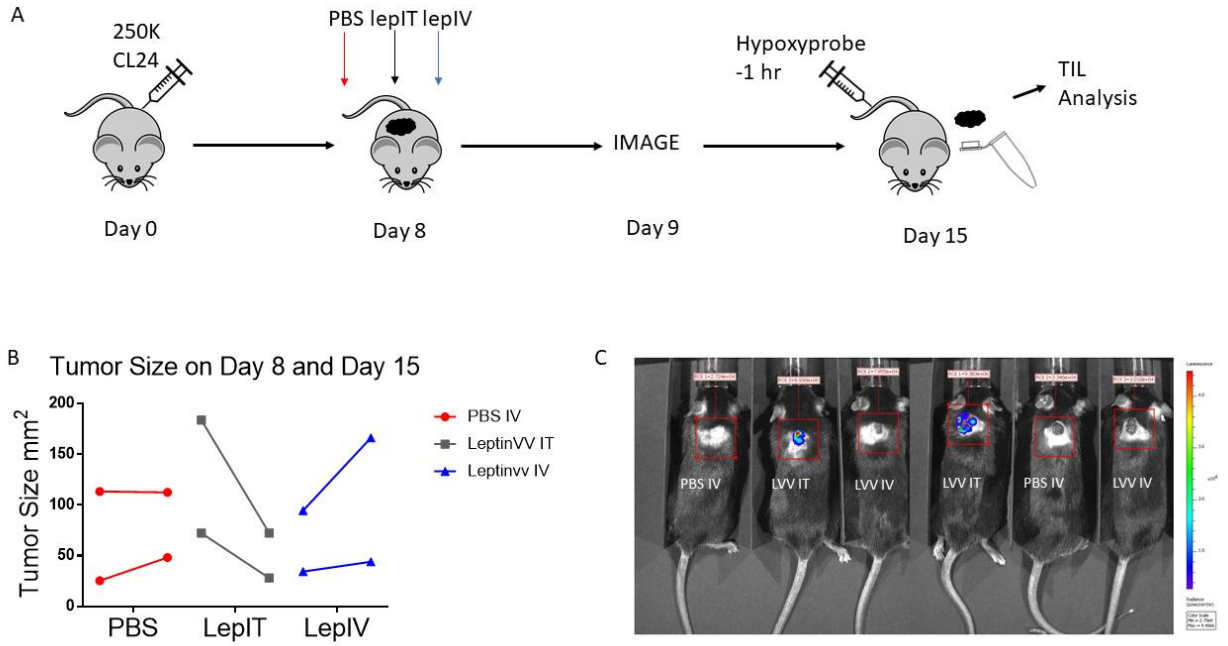

Figure 1. **Tumor treatment, growth, and imaging**, *a*. Experimental outline scheme *b*. Tumor measurements on day 8 (treatment day) and day 15 (harvest) *c*. Imaging the mice on the IVIS for presence of luminescent virus

Flow cytometry analysis of TIL

Analysis by flow cytometry showed very low levels of hypoxyprobe staining in all mice (data not shown). As a result of such low staining in the tumor, which should have bright hypoxyprobe positive stained cells, data was analyzed without regard to hypoxyprobe staining. There was little to no change in the percentage of infiltrating T cells with either leptin-vv treatment as compared to the PBS treatment (Figure 2a). This corresponds to previous data showing that leptin does not increase tumor infiltration by CD8 T cells. There is higher Ki67+ expression in unstimulated CD4 and CD8 T cells in the intratumorally treated animals (Figure 2b). Increased Ki67+ T cells in after leptin treatment has also been previously described. Expression of Ki67 increases after stimulation only in the CD8+ T cells of the intravenously treated animals.

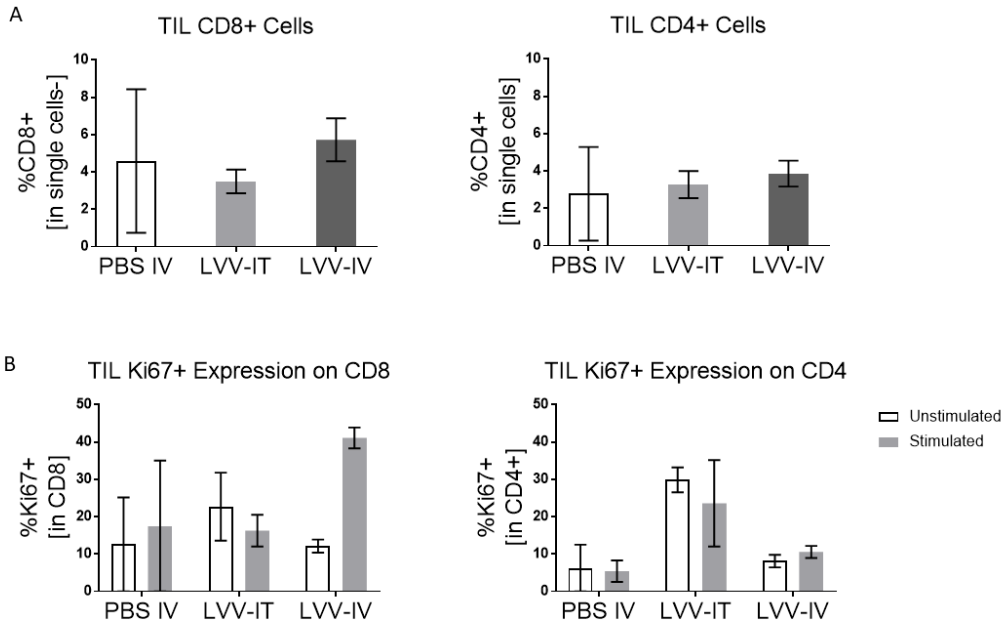

Figure 2. **T cell populations in the tumor,** Flow cytometry was used to determine the presence of T cell populations in the tumor *a.* Quantified percentages of CD8+ cells *b.* Quantified percentages of Ki67+ CD8+ cells *c.* Quantified percentages of CD4+ cells *d.* Quantified percentages of Ki67+ CD4+ cells

The tumor infiltrating cells with both IT and IV injection have a different exhaustion marker profile than the PBS treated mice. Most evident is the emergence of a TIM3+ PD1- population which is larger in the IT treated mice than the IV (Figure 3a). The double positive cells also increased with treatment. In each treatment condition tumor infiltrating CD8+ T cells were able to produce IFNγ after stimulation however, production was highest in the PBS treated mice (Figure 3b). There is a trend towards a further decrease in the IV treated mice over the IT treated mice. The IV treated mice had fewer Tregs in the TIL than the IT or PBS treated animals (Figure 3c). There was no increase in glucose uptake with either treatment condition (Figure 4a). The leptin-vv IV treated animals had an increased mitochondrial mass than the leptin-vv IT or the PBS treated animals (Figure 4b). Due to the low staining of mitotracker on CD8+ T cells in the lymph node however the mitotracker staining cannot be relied upon as these T cells should be mitotracker high (Figure 4c).

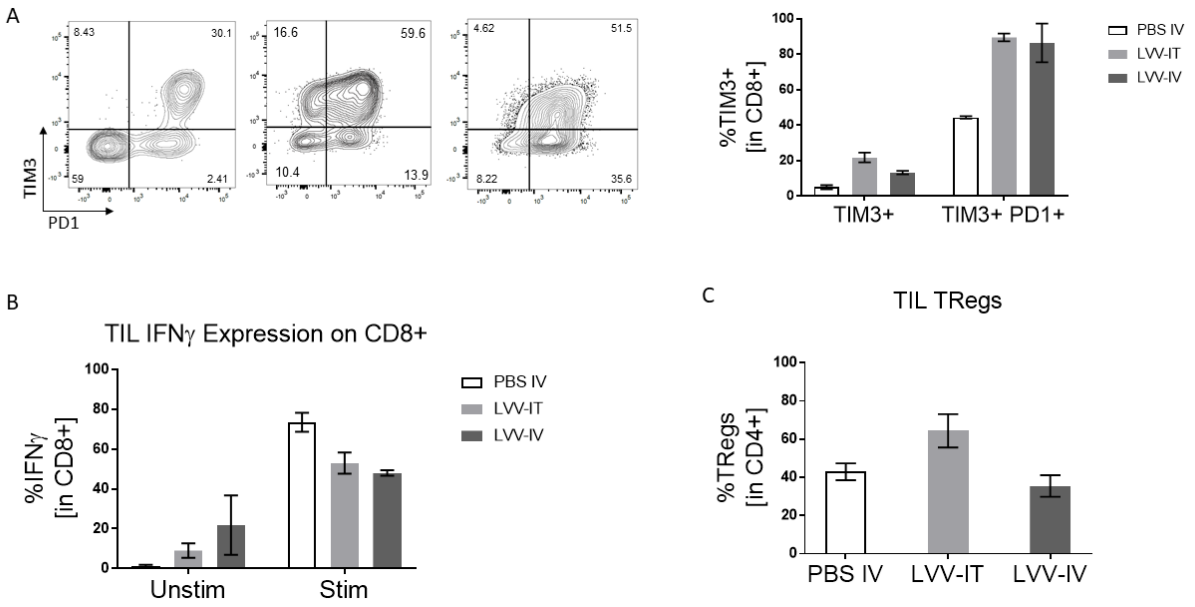

Figure 3. **Exhaustion of Tumor Infiltrating T cells ,** Flow cytometry was used to determine the presence of T cell populations in the TIL *a.* Contour plots of representative samples and quantified percentages of TIM3+ and TIM3+ PD1+ CD8 T cells *b.* Quantified percentages of IFNγ producing CD8+ T cells *c.* Quantified percentage of T-regs

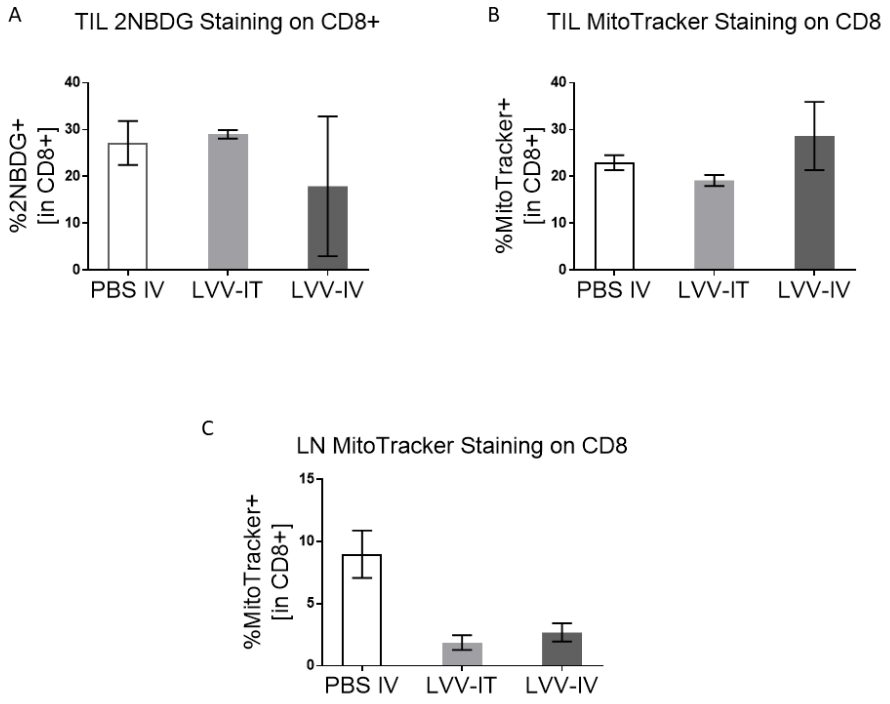

Figure 4. **Metabolic ability of CD8+ T cells in the tumor,** Flow cytometry was used to determine glucose uptake or mitochondrial mass in the TIL and lymph node *a.* Quantified percentages of 2NBDG staining on CD8 T cells *b.* Quantified percentages of mitotracker staining on CD8 T cells *c.* Quantified percentages of mitotracker staining in the lymph node

DISCUSSION

Tumor growth was slowed only in the leptin-vv intratumoral animals which may be a result of insufficient virus reaching the tumor in the IV group as virus was not visualized in these animals. This could be a result of imaging at the wrong time point. In the intratumoral route of injection the virus is delivered directly to the tumor and it can quickly begin replicating. In the intravenous route of injection the virus must travel to the tumor and then begin replication. It is possible that this takes longer than 24 hours to begin and should prompt imaging of IV treated animals at longer time points after treatment. It is also known that there may be an anti-viral response to oncolytic viruses which may prohibit the virus from reaching the tumor. Further studies will be needed to determine if both the innate and adaptive anti-viral responses are preventing the virus from reaching the tumor. It is also important to note that while no virus was imaged in the IV treated group, immune changes were observed that matched those observed in the IT group, leading us to believe virus did eventually reach the tumor.

There is no major change in T cell infiltration into the tumor with either leptin-vv treatment. There is a higher percentage of Ki67+ CD4+ T cells in the intratumoral treated mice that was not observed in the IV treated mice. This suggests that proliferation of CD4+ T cells may increase after treatment with the leptin-vv.

No changes were seen in the metabolism of the CD8 T cells as shown by 2NBDG staining. While it appeared there may be a slight increase in mitochondrial mass via mitotracker staining it was disregarded as mitotracker was stained poorly in the lymph node. As these lymph node CD8+ cells are not experiencing the stress of the tumor microenvironment they should have high mitotracker staining.

The exhaustion profile of the CD8+ tumor infiltrating cells was changed with leptin-vv treatment. Both IV and IT treated animals had a TIM3+ PD1- population appear that was not present in the PBS group. This population may represent a more activated T cell. In the future these TIM3 single positive cells could be sorted and studied for phenotypic differences from traditional $PD1^{hi}$ exhausted CD8+ cells. The leptin-vv animals also had a shift towards a decrease in PD1 single positive cells from $PD1^{hi}$ to $PD1^{mid}$, a less exhaustive phenotype. There is also an increase in the exhausted PD1+ TIM3+ CD8 T cells in these animals. The appearance of $PD1^{mid}$ and TIM3+ PD1- CD8 T cells coupled with a decrease in ability of CD8+ cells to produce IFNγ suggest that while there are indeed exhausted T cells in this microenvironment the leptin-vv may be lessening this exhausted phenotype in the tumor. As we see no increase in CD8+ cells with treatment these are most likely not newly recruited cells. Moving forward there may be potential for combination therapy with leptin-vv and αPD1 as a way to trigger these less exhausted cells to target the tumor for lysis. It is also possible that while we do not see increased numbers of CD8+ T cells in the TIL new cells are being actively recruited, however through innate mechanisms triggered by the virus such as macrophage activation old cells are cleared, leaving the total percentage unchanged. More work needs to be done to clarify the role each of these populations is playing in the anti-tumor immunity generated by the leptin-vv.

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