Leptin Repolarizes Tumor-Associated Macrophages to Boost Immunotherapy Efficacy in Obesity

By

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This work is dedicated to my mom, my husband, and my son who always supported me through the ups and downs and celebrated every small victory in lab and in life with me.

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I. INTRODUCTION

Background and Significance

History of Obesity and Cancer Associations

The link between obesity and "karkinos," now known as cancer, dates back to Hippocrates (460-370 BC), where the "Father of Medicine" connected overeating and sedentary lifestyle to cancer.[1] While the initial association was made early in the history of medicine, it took almost two millenniums for the next scientist, Robert Thomas, to associate endometrial cancer with obesity.[2] Initially, to understand the cause of increased cancer risk in obesity, scientists investigated local interactions between adipose tissue and tumor development such as infection or tissue damage. Scientists discovered that obese adipose tissue was filled with immune cells and induces inflammation, where the greatest inflammation occurs in visceral adipose tissue. Subsequently, obesity was identified as a risk factor for esophageal adenocarcinoma, colon cancer, renal cell cancer, postmenopausal breast cancer, endometrial cancer, and prostate cancer, which are all surrounded by visceral adipose tissue.[3–6] However, as our understanding of the systemic effects of obesity have increased so have the number of cancer subtypes associated with obesity. Today, obesity is associated with 13 different types of cancer.[7] In the United States 34.9% of adults are obese (BMI $\geq$ 30.0) and 68.3% are overweight (BMI 25.0-29.9 kg/m^2).[7,8] Worldwide, 1.9 billion adults are overweight with 650 million meeting obesity criteria, and in children, 340 million age 5-19 years old are obese and an additional 40 million children under 5 years old are obese.[9] It is estimated that obesity is the cause of 14% and 20% of cancer deaths in men and women, respectively.[10,11] Intestinal cancers, such as esophageal, stomach and

colorectal have the highest relative risk with obesity.[10] Despite a decline in the overall rates of cancer diagnoses, particularly those associated with tobacco, the rates of obesity-associated cancers are increasing and are expected to surpass tobacco as the number one preventable risk factor for cancer.[11–15] Along with an increased risk of developing cancer, obese patients also have worse outcomes and responses to surgery, chemotherapy, and radiation therapy treatments.[16]

Hormone Changes in Obesity

Initially, adipose tissue was viewed as static tissue whose job was to store excess lipids, mostly triglycerides. As adipose tissue increases in obesity, the levels of adipokines change as well, affecting people systemically. Many obese people who develop cancer have metabolic syndrome and insulin resistance as a precursor. Insulin resistance is defined by elevated blood glucose concentrations while fasting, impaired glucose tolerance, elevated levels of insulin while fasting, and decreased insulin sensitivity. The leptin hormone, which is produced by adipocytes and signals satiety, is also elevated in obesity.[17] The elevated leptin levels also ultimately result in "leptin resistance" just as the elevated insulin results in insulin resistance.[18] The last main metabolic hormone change in obesity is a decrease in adiponectin, which is an insulin-sensitizing adipokine.[19,20] High levels of adiponectin are associated with a decreased risk for obesity-associated cancers, while a high leptin to adiponectin ratio increases the risk for obesity-associated cancers.[21–25] Since peripheral tissues do not respond to normal levels of insulin hormone, pancreatic β-cells increase their insulin production to compensate for the insulin resistance, which causes elevations in insulin-like growth factor-1 (IGF-1).[26] The increase in signaling through the insulin receptor and IGF-1 receptor is correlated with an increased risk of cancer.[27,28]

Immune Cells in Adipose Tissue

The past few decades have been pivotal in understanding the interactions between adipocytes and immune cells in the adipose tissue, and how the resident immune cells, adipokines, and hormones change in that of lean versus obese adipose tissue. While adipocytes are the largest component of adipose tissue by volume, immune cells can greatly outnumber adipocytes by cell number. In adipose tissue of the lean population, the overall immune cell phenotypes have an anti-inflammatory phenotype. The predominate T cell subtypes that prevent inflammation in lean adipose tissue are T regulatory cells (Tregs) and T_H2 helper T cells (**Figure 1.1**).[29,30] The healthy adipose and endothelial tissue secretes interleukin 33 (IL-33), which retains a significant population of T regulatory cells (Tregs).[31–33] The Tregs in turn secrete interleukin 10 (IL-10), which is the strongest immunosuppressive cytokine. Additionally, the Tregs interact with innate lymphoid 2 (ILC2) cells, resulting in ILC2 release of interleukin 5 (IL-5) and interleukin 13 (IL-13) cytokines, sustaining eosinophils and M2-like macrophages, respectively.[34] The eosinophils produce interleukin 4 (IL-4) and T_H2 helper T cells produce IL-4 and interleukin 12 (IL-12), further promoting an anti-inflammatory M2-like macrophage phenotype (**Figure 1.1**).[35–38] The lean adipose tissue also secretes high levels of adiponectin, which inhibits proinflammatory immune cell function and is associated with decreased cancer risk.[23,39]

However, as adipose tissue accumulates, the adipocytes and endothelial cells secrete monocyte chemoattractant protein-1 (MCP-1) and leukotriene B4, attracting monocytes and neutrophils into the adipose tissue, respectively (**Figure 1.1**).[31,40] Additionally, the increased number of adipocytes increase levels of the leptin hormone. In the presence of major histocompatibility complex II (MHCII) on adipocytes and myeloid

cells, the leptin promotes a T_H1 phenotype of $CD4^+$ helper T cells.[35] The chemoattracted neutrophils release elastase, promoting interferon-gamma (IFNγ) release from cytotoxic $CD8^+$ T Cells, T_H1 type $CD4^+$ helper T Cells, and natural killer cells (**Figure 1.1**).[41] The IFNγ plays a dual role in promoting pro-inflammatory inflammation through polarization of macrophages to an M1 phenotype and further MHCII upregulation on adipocytes, while also inhibiting Tregs.[35,36,42] Mice that are deficient in IFNγ have decreased adipose-tissue inflammation and immune cell infiltration.[43] The IFNγ stimulates nuclear-factor kappa-light chain enhancer of activated B cells (NFκB) and polarizes macrophages to an M1 phenotype or classically activated phenotype. B cells are also recruited to adipose tissue early in the accumulation of adipose tissue, and the B cell antibody production contributes to the inflammatory state. Of note, interaction between the B cells and T cells is required for insulin resistance to develop, as B cells deficient in antigen presentation do not cause insulin resistance.[44] Obese adipose tissue also contains an increased number of mast cells, which increase systemic levels of tryptase, and depleting mast cells reduces overall systemic inflammation.[45] This feed forward mechanism of pro-inflammatory immune cell recruitment and systemic pro-inflammatory cytokines produces a vicious cycle of sustained, chronic inflammation that results in a concomitant of comorbidities for obese patients.

Immune Cell Changes in Obese Adipose Tissue

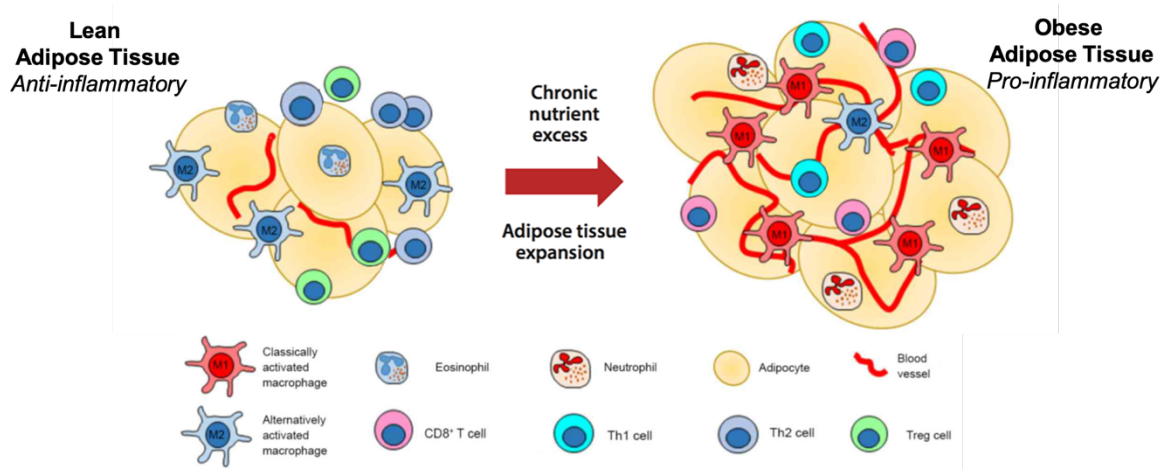

Figure 1.1. Lean adipose tissue has small adipocytes that are surrounded by anti-inflammatory immune cells including alternatively activated M2-like macrophages, eosinophils, T Regulatory (Treg) cells, and T_H2 T cells. In obesity, the adipocytes increase in size and number and secrete a variety of chemokines and adipokines that attract pro-inflammatory immune cells including classically activated M1-like macrophages, T_H1 T cells, $CD8^+$ T Cells, and neutrophils.[46]

The Role of Macrophages in Obesity-Induced Inflammation

In 2003, scientists discovered that the adipose tissue macrophages (ATMs) are the most important cell in producing adipose-tissue inflammation, and subsequently systemic inflammation in obesity.[47] In lean adipose tissue, the macrophages are found between the adipocytes and blood vessels, and these macrophages have an "M2" phenotype whose function is to support homeostasis of the adipose tissue (**Figure 1.2**). This is achieved through several mechanisms including promotion of angiogenesis and remodeling extracellular matrix in the adipose tissue.[48,49] An M2 macrophage (alternatively-activated) increases insulin sensitivity and promotes wound healing or repair through activation of peroxisome proliferation activated receptors (PPAR) γ , PPAR δ, and activator of transcription 6 (STAT6) and subsequent production of IL-10 and arginase.[50–52] By histology, these M2 macrophages can be identified with a CD206 mannose-receptor

antibody (**Figure 1.2**). While CCR2 recruits macrophages in obesity, the M2 macrophages in lean adipose tissue are recruited in a CCR2/CCL2 independent mechanism.[38] As adipocytes enlarge and enumerate in obesity, a "phenotypic switch" begins in the macrophages, where recruited M1 macrophages outnumber the resident M2 macrophages, particularly in the visceral adipose tissue (**Figure 1.2**). The recruitment of M1 macrophages occurs quickly, and the majority of the M1 macrophages concentrate into crown-like structures around dead adipocytes.[38,42] On histology, the M1-macrophages appear to be filled with fat or lipid-laden as seen in atherosclerotic foam cells, and the M1 macrophages are positive for both CD206 and CD11c.[53,54] The recruited M1 macrophages contribute to adipose tissue inflammation via their secretion of pro-inflammatory cytokines interleukin-1 beta (IL-1β), interleukin-6, (IL-6), interleukin-8 (IL-8), MCP-1, inducible nitric oxide synthase (iNOS), and tumor necrosis factor-alpha (TNFα), where TNFα contributes directly to insulin resistance (**Figure 1.2**).[47,55–59] Along with cytokines, obesity induces an increase in $CD11b^{+}Ly6C^{+}$ circulating monocytes.[60] In other tissues, M1 macrophages arise due to exposure to lipopolysaccharides (LPS) from bacterial infection and IFNγ, in which the M1 macrophage's secretion of pro-inflammatory cytokines (iNOS and TNFα) support pathogen killing and serve a function to protect the body. To produce these pro-inflammatory cytokines, the M1 macrophages increase glucose and iron uptake, while decreasing fatty acid oxidation.[61] Comparatively, M2 macrophages use fatty acid oxidation for energy and release iron.[62] M1 macrophages also contribute to adipocyte dysfunction and impair adipogenesis, further inhibiting fatty acid storage and disrupting adipose tissue function.[63] The prevention of lipid storage in adipose tissue forces lipids to be stored in other tissues, such as the liver, and can lead to organ dysfunction.

Macrophage Polarization Spectrum

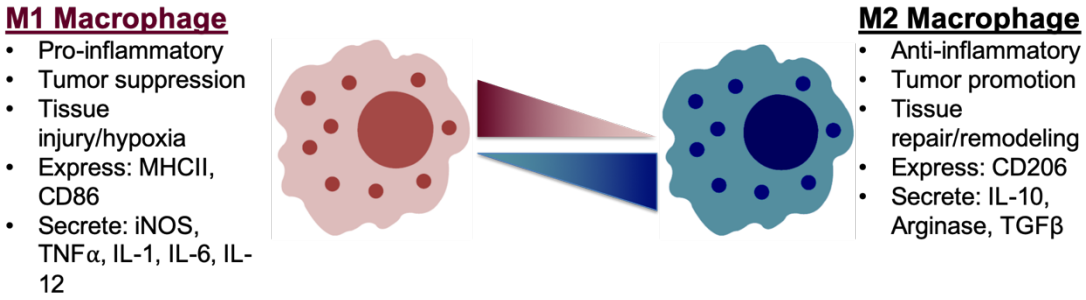

Figure 1.2. Macrophages can lie anywhere on the macrophage polarization spectrum. The M1 macrophage's pro-inflammatory function is ideal for bacterial infections or tumor suppression. The other end of the spectrum has the M2 anti-inflammatory macrophages, which play a role in wound healing and tumor promotion. However, macrophages can have co-expression of M1 and M2 surface markers or secrete products made by M1 and M2 macrophages.

The accumulation of pro-inflammatory immune cells ($CD8^+$ T cells, T_H1 $CD4^+$ helper T cells, M1-like macrophages, and B cells) and their secreted cytokines contribute to insulin-resistance. While all immune cells contribute, the macrophage is the strongest contributor to inflammation and insulin resistance.[33,64,65] The TNFα secreted by macrophages activates a variety of serine kinases including IκB kinase (IKK), c-Jun N-terminal kinase (JNK), S6 kinase (S6K), mammalian target of rapamycin (mTOR), and double-stranded RNA-dependent protein kinase (PKR).[66–70] These kinases phosphorylate insulin-receptor substrate 1 (IRS1) on serine residues, which decreases insulin signaling. Mouse experiments that depleted $CD8^+$ T cells or B cells, both showed improved insulin sensitivity.[44,71] When blocking the IKKβ signaling pathway in mice, there is a decreased in pro-inflammatory cytokine production from macrophages, and the mice remain insulin-sensitive.[72,73] Using a mouse model that was leptin deficient and over expressed adiponectin, the diet-induced obese mice had increased subcutaneous fat without an increase in adipose tissue macrophages, no increase in visceral adipose tissue, and no development of metabolic syndrome.[22]

Adipocyte Induced Inflammation

Additional components of the adipose tissue that are not immune cells also contribute to excess inflammation and insulin resistance. As the adipose compartment expands with an increasing number of adipocytes, a large number of the adipocytes undergo apoptosis, which recruits additional immune cells.[42] The adipocytes undergoing apoptosis lie adjacent to macrophages, and by histology, the white adipocyte with macrophage stain surrounding it is known as a "crown-like structure."[42] These "crown-like structures" have been associated with insulin resistance and metabolic syndrome.[53,74–76] Adipocyte lipolysis releases free fatty acids (FFAs) that contribute to insulin resistance.[77] Saturated fatty acids (SFAs) are capable of activating the pattern recognition receptors (PRRs) Toll-like receptor 4 (TLR4) and Toll-like receptor 2 (TLR2), contributing to further inflammation, although the activation may be indirect.[78–82] The typical activator of TLRs is pathogen-associated molecular patterns (PAMPs) from bacteria, such as lipopolysaccharides (LPS), and LPS is often used to produce M1 pro-inflammatory macrophages *in vitro*. SFAs are also capable of increasing pro-inflammatory genes and cytokines through inflammasome activation and altering cell membranes, which increases JNK signaling and proinflammatory genes.[83,84] Ceramide synthesis is also increased directly via SFA metabolites, and elevated intracellular ceramide increases insulin resistance by inhibiting insulin signaling via Protein kinase B (PKB/Akt) inhibition.[85–87] SFA also increases ceramide synthesis indirectly through TLR4 activation.[85,88] Studies have shown that inactivation of TLR2 or TLR4 receptor inhibits the development of insulin resistance in diet-induced obese mice.[89–91] While the adipose tissue gains adipocytes, it does not vascularize appropriately, resulting in areas with insufficient vasculature and subsequent hypoxia.[92,93] Production of hypoxia-inducible factor (HIF) occurs in the

hypoxic tissue, and HIF-1α activates pro-inflammatory cytokines, resulting in further insulin resistance.[94] Together, these changes create elevated systemic glucose, insulin, IGF-1, leptin, and pro-inflammatory cytokines with decreased adiponectin.

Of note, obesity can occur without chronic inflammation. In the obese population, 20-30% do not have an inflamed profile either in adipose tissue or systemic circulation, nor do they have metabolic syndrome with insulin resistance.[75,95] The discrepancy between high BMI and low inflammation in this minority of obese patients is still being investigated, but people with a high BMI, low muscle mass, and greater visceral adiposity (abdominal obesity) instead of subcutaneous adiposity have increased risk for developing metabolic syndrome and obesity-associated inflammation.[96–100] It is hypothesized that the non-inflamed obese population does not have an increased risk of developing obesity-associated cancers.[101] Additionally, in the elderly population, "unhealthy aging" may occur in which there is an association of pro-inflammatory but often senescent cells and pro-inflammatory cytokines coupled with muscle wasting/sarcopenia and frailty, creating an increased risk for metabolic syndrome.[102–105]

Mechanisms for Obesity Promoting Cancer

Obesity induces a variety of changes in the body, both locally in adipose tissue as well as systemically, and these changes contribute to all of Hanahan and Weinberg's original "Hallmarks of Cancer."[106] For the hallmarks that involve proliferation: sustained proliferative signaling, resisting cell death, and enabling replicative immortality, metabolic and immune associated changes promote pro-survival cytokines and decrease apoptotic gene expression.[107] Genomic instability is another hallmark of cancer that is linked to obesity.[108] When genomic instability occurs, there is an increased risk to acquire mutations, and this is classified as either microsatellite instability (MIN) or chromosomal instability (CIN).[108] A subtype of MIN is microsatellite-instable (MSI), and obesity increases the risk of MSI-high or MSI status in colorectal cancer and endometrial cancer, respectively.[109,110] CIN risk is also increased in the obese population with endometrial cancer, connecting obesity to both types of genomic instability.[111]

While obesity was associated with many of Hanahan and Weinberg original

"Hallmarks of Cancer," obesity also contributes to the "next generation" of hallmarks, but the mechanism of these associations are still poorly understood.[106,108] The combination of immune and hormonal changes in obesity induce specific genetic changes that created an "obesity" gene signature, which was associated with decreased time to metastasis in breast cancer patients.[112] The obesity signature genes identified from whole-genome analysis in over 1500 patients including those associated with insulin growth factor (IGF) signaling and decreased estrogen receptor expression.[112] Despite these genetic changes, protein microarray data from obese and lean patient tumor samples have not identified any differences, limiting the translational impact where genetic differences do not result in functional tumor differences.[113]

The chronic-low grade inflammation in obesity can be associated with multiple mechanisms of tumor initiation and progression, depending on cancer subtype.[114–118] Carcinogenesis via inflammation may occur from deoxyribose nucleic acid (DNA) damage, genomic instability, enhanced proliferation of cells, and resistance to apoptosis. The signaling in chronic inflammation activates STAT3, which increases proliferation and decreases apoptosis in all cells, not just immune cells.[119] Similarly, NF-κB is also upregulated in chronic inflammation, further increasing proliferation and pro-survival gene expression in cells.[120] Both STAT3 and NF-κB contribute to the original hallmark of activating invasion and metastasis through their implication in EMT.[106] The increase in inflammation and proliferation from STAT3 and NF-κB is associated with an increase in genomic instability, broken DNA repair mechanisms, and subsequent increased rates of DNA damage, which creates cells that are more invasive and have greater metastatic potential.[121,122] While there are many cytokines elevated in obesity that induce chronic

inflammation (MCP-1, IL-6, TNFα, etc.), no studies to date have pinpointed a specific cytokine as a biomarker for all obesity-associated cancers, and ongoing studies are trying to establish the mechanisms associating chronic inflammation with cancer development.[108]

Despite good scientific evidence detailing how obesity results in chronic inflammation, there is limited literature investigating the relationship between adipose tissue macrophages and tumor-associated macrophages in obesity.[123] Studies comparing peritumoral fat and non-malignancy associated fat have started to unravel how chronic inflammation in obesity supports cancer development. For example, while adipose tissue in obesity typically has pro-inflammatory cells and genes, adipose tissue surrounding breast tumors has increased levels of anti-inflammatory genes and genes that support proliferation, invasion, and angiogenesis.[124] This results in larger mammary tumors in DIO mice that have increased numbers of both TAMs and hypertrophic adipocytes compared to lean mouse mammary tumors.[125] Similarly, DIO mice with ovarian tumors have an increased M2:M1 macrophage ratio and increased tumor vascularity compared to lean mice with ovarian tumors.[126] Overall, the current studies suggest that the tumor stroma and surrounding adipose tissue in obesity support an increased M2-like tumor-associated macrophage phenotype compared to tumor-associated macrophages in lean subjects.[108]

Tumor-Associated Macrophages and Cancer

Tumor-associated macrophages (TAMs) usually make up the greatest portion of immune cells in tumors, and these macrophages typically have an M2 phenotype that supports tumor growth and inhibits anti-tumor immune cells.[127–132] Tumor and tumor-stromal cells produce the growth factors: macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which increase the

production of monocytes in the bone marrow and circulating $CD11b^{+}Ly6C^{+}CCR2^{+}$ monocytes migrate into the tumor often before lymphocytes infiltrate the tumor.[127,133,134] The local M-CSF induces macrophage maturation, and high levels of M-CSF are associated with a poor prognosis in ovarian, breast, and endometrial cancer.[135] The M-CSF, prostaglandin E-2 (PGE2), TGFβ, IL-6, and IL-10 in the tumor microenvironment polarize TAMs towards an M2 phenotype. In turn, the TAMs promote tumor progression and angiogenesis, tumor invasion and metastases, and immunosuppression. While the majority of TAMs have this M2-like phenotype, TAMs are capable of a variety of functions and phenotypes, dictated by tumor type, stage, and location.[136–138] Despite this, when macrophages are depleted in tumor models, the tumors progress and invade at a slower rate than control tumors.[139]

TAMs significantly increase metastatic risk by promoting all steps required for metastasis, increasing their association as a negative prognostic factor for patient survival as more than 90% of patients die from metastatic disease.[140,141] Growth factors secreted by TAMs which include epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), directly support tumor cell proliferation. When TAMs mediate ECM degradation via matrix metalloproteinases (MMPs), additional growth factors are secreted.[142,143] TNFα secreted by TAMs increases transcription of nuclear factor-κB (NF-κB) in tumor cells, promoting proliferation, while TAM-secreted IL-6 activates STAT3 and induces genes that promote cell cycle progression (Ex: cyclin D) and suppress apoptosis (Bcl-2).[144] TAMs contribute to another characteristic of tumors, which is their disruption of tissue architecture that allows for increased invasion into blood vessels and metastases. While TAMs secrete a different profile of ECM proteins than that of benign tissue, TAMs protease secretion including MMPs and cathepsin breaks down

physical barriers between cells, allowing for tumor invasion into adjacent tissues.[145] TAMs also support epithelial-to-mesenchymal transition (EMT) through their TGFβ production, as mice with TGFbr2 deletions had decreased rates of metastases.[146] Tumor cells perform more glycolysis than normal cells, regardless of oxygen availability (Warburg effect).[147] Macrophages are recruited to and accumulate in hypoxic areas of the tumor due to hypoxic-inducible factor alpha (HIF-1α) signaling pathway, and the TAMs promote angiogenesis through VEGF secretion.[148,149] Clinically, hypoxia is associated with aggressive tumor proliferation and increased metastatic risk.[150,151]

Tumors promote an immunosuppressive phenotype, and one recruited cell type that promotes this is the myeloid-derived suppressor cell (MDSC). MDSCs are immature cells that suppress cytotoxic T-cell activation and promote T regulatory cells, and MDSCs are classified as either granulocytic-MDSCs (G-MDSCs) or monocytic-MDSCs (M-MDSCs).[152,153] MDSCs directly interact with TAMs and promote an M2-polarization via IL-10 production, which decreases IL-12 macrophage production (**Figure 1.3**).[154] M2-polarized TAMs themselves secrete numerous cytokines that inhibit cytotoxic T cells and natural killer cells including, IL-10, IL-4, IL-6, PGE_2, TGFβ, and CSF-1.[155] TAMs also inhibit dendritic cell tumor-antigen presentation from its IL-10 and VEGF cytokine production, thereby inhibiting adaptive immunity.[155] Obesity increases MDSC accumulation in tumors, creating a potential for stronger M2-polarization of TAMs in obese subjects versus lean subjects (**Figure 1.3**).[156]

Obesity Increases Myeloid-Derived Suppressor Cells and M2-like Macrophages

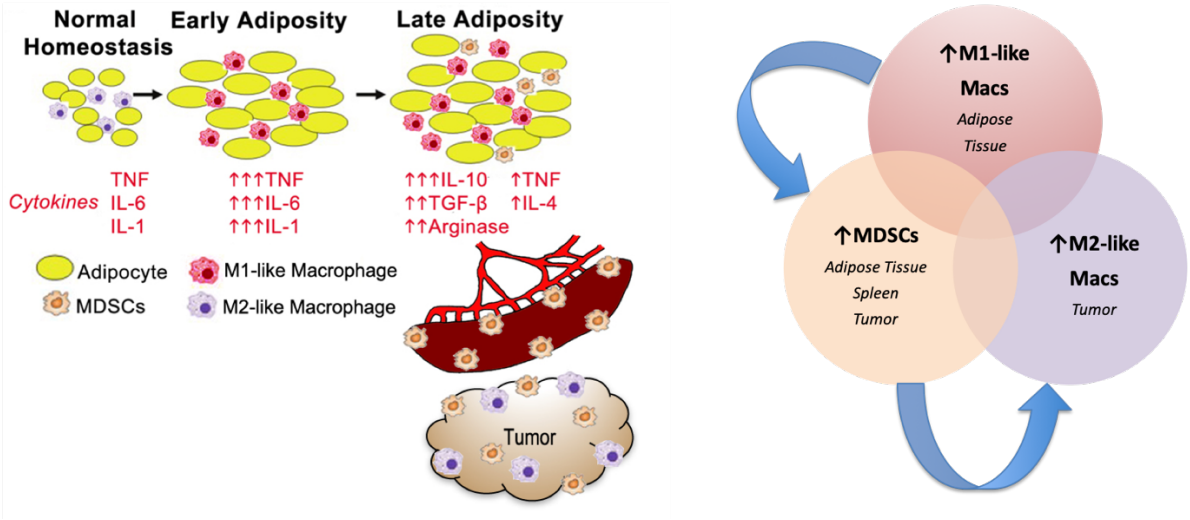

Figure 1.3. In a lean state, the small adipocytes are surrounded by M2-like macrophages and other anti-inflammatory immune cells that maintain homeostasis. As adipose tissue increases, the size and number of adipocytes increases, resulting in an increase in M1-like macrophages that increase TNF, IL-6, and IL-1 cytokines. When an obese state persists, myeloid-derived suppressor cells (MDSCs) accumulate in the adipose tissue, as well as other organs like the spleen and liver. The secretion of cytokines such as IL-10 by MDSCs aims to decrease the overall systemic inflammation. However, when a tumor also occurs in this setting, the MDSCs also accumulate in the tumor. The cytokine secretion from MDSCs in tumors support a stronger M2-like tumor-associated macrophage (TAM) than in lean subjects. The stronger M2-like TAM burden in obesity supports tumor growth and increase the risk for metastatic potential.[157]

Since TAMs play a large role in tumor progression and immunosuppression, studies investigating drugs that remove or repolarize TAMs demonstrate decreased tumor burden and metastatic rate. CSF-1 receptor inhibitors induce decreased angiogenesis and metastasis rates in myeloid leukemia and melanoma, while anti-CSF-1 antibodies and antisense treatments decrease TAMs in mouse models of breast cancer.[158–160] Antibodies against MCP-1 and VEGF inhibitors have also been shown to decrease TAM recruitment, and these treatments were transitioned to clinical trials.[134,161,162] When repolarizing TAMs towards a M1-phenotype with IFNγ or CD40 agonist antibodies, increased MHCII expression and tumoricidal activity was observed.[163,164]

Cancer Immune Checkpoint Blockade

In normal physiology, the purpose of immune checkpoint upregulation is to decrease systemic inflammation in order to prevent the development of autoimmune disease. However, tumors will also induce upregulation of immune checkpoints to inhibit the host's immune cells from attacking tumor cells. The immune checkpoint that inhibits early antigen presentation in lymph nodes is cytotoxic T lymphocyte associated protein 4 (CTLA-4). When antigen presentation occurs, an antigen presenting cell's HLA must present the antigen to TCR on a T cell, but co-stimulation must also occur through the interaction of CD80/CD86 on an antigen presenting cell and CD28 ligand on the T cell. When CTLA-4 is upregulated on T cells, it binds to CD80/CD86, inhibiting co-stimulation and preventing antigen presentation. Locally in the tumor, tumors can upregulate programmed cell death ligand 1 or 2 (PD-L1/B7-H1 or PD-L2/B7-DC), while immune cells may upregulate programmed cell death-1 (PD-1). The interaction between PD-1 and PD-L1 induces an exhausted T cell phenotype from inhibition of tumorigenic killing by cytotoxic T cells, decreasing T cell proliferation, and decreasing anti-tumor cytokine production, such as IFNγ (**Figure 1.4**). A high PD-L1 expression in tumors is associated with poor differentiation of the tumors and poor patient prognosis in non-small cell lung cancer (NSCLC) and melanoma.[165–167] When administering anti-CTLA-4 antibodies or anti-PD-1 antibodies, the interactions between CTLA-4/CD28 and PD-1/PD-L1 are blocked, and immune cell function is rescued (**Figure 1.4**). This allows T cells to attack tumor cells and can lead to decreased tumor progression and even tumor regression in patients.[168,169]

Immune Checkpoint Blockade Antibody Mechanism

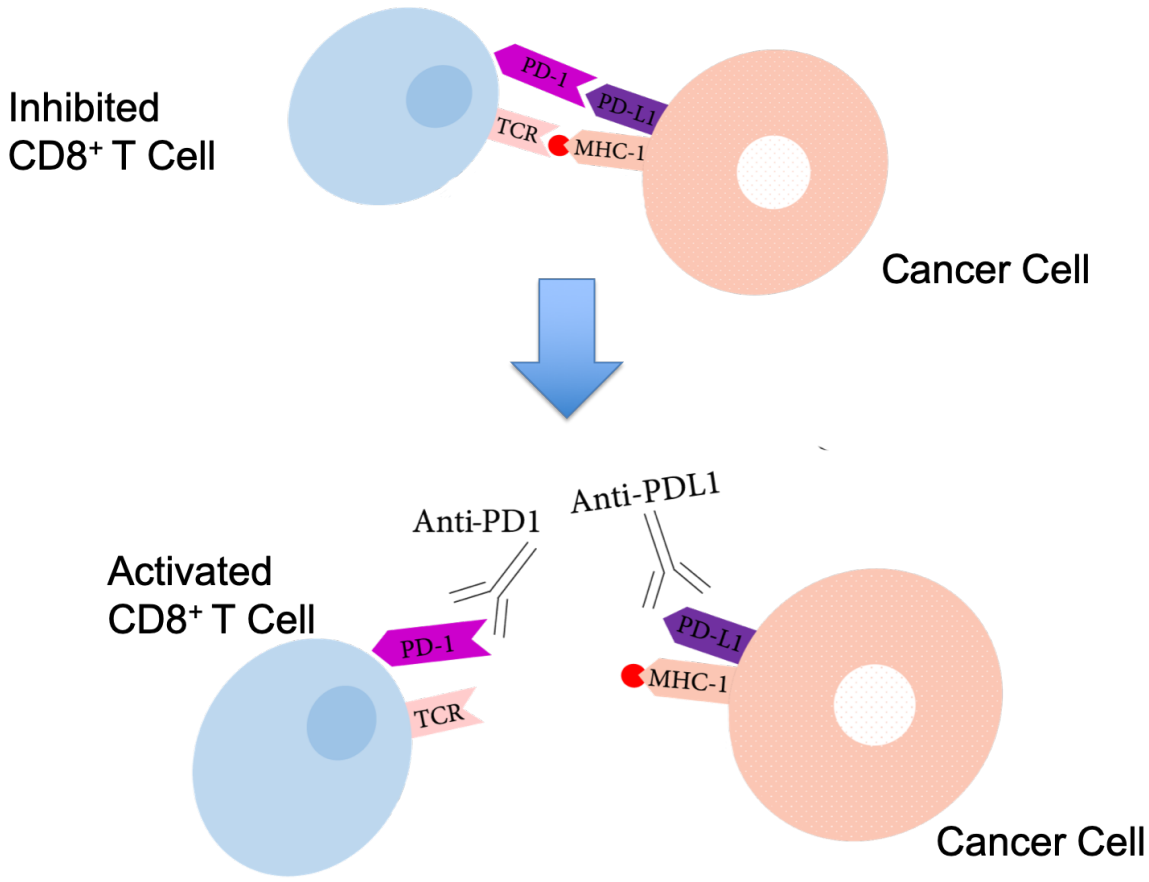

Figure 1.4. The immune checkpoint blockade antibodies allow a patient's own immune cells to regain their function and attack the cancer cells. The T cell receptor on a $CD8^+$ T cell recognizes a tumor antigen expressed on the cancer cell's MHC-I receptor. However, the PD-1/PD-L1 expression and interaction by $CD8^+$ T cell and cancer cell, respectively, prevents the $CD8^+$ T cell from killing the cancer cell. When treating with an anti-PD-1 or anti-PD-L1 antibody, the PD-1/PD-L1 axis is blocked and the $CD8^+$ T cell's effector function is restored.[170]

Predicting Responders to Immune Checkpoint Blockade

Immune checkpoint blockade treatments have shown increased patient survival in many types of cancer including melanoma, lung cancer, bladder cancer, renal cancer, colon cancer, and more.[171] The PD-1/PD-L1 checkpoint blockade treatments have shown increased overall survival with a decrease in side effects for patients, especially in melanoma and NSCLC.[171–174] However a minority of patients respond to these treatments,

such as less than 20% of NSCLC patients responding to anti-PD-1 antibodies in the KEYNOTE001 trial (**Table 1.1**).[175] This has sparked a widespread effort by clinicians and researchers to determine what the best biomarkers are for predicting response to immune checkpoint blockade treatments.

Immune Checkpoint Blockade Efficacy

Tumor Type	Number of Patients	Response Rate
Colon Cancer (Mismatch repair-deficient/ Microsatellite Instable)	74	31%
Melanoma	107	31%
Renal Cell Caner	34	29%
Non-Small Cell Lung Cancer	127	16%

Table 1.1. Response rates for colon cancer(mismatch repair-deficient and microsatellite instable), melanoma, renal cell cancer, and non-small cell lung cancer patients treated with anti-PD-1 Nivolumab as a monotherapy.[176,177]

Knowing that anti-PD-1 and anti-PD-L1 antibodies inhibit interaction between the PD-1/PD-L1 axis, expression of PD-L1 on tumors and PD-1 on T cells were among the first biomarkers investigated.[171] Immunohistochemistry (IHC) was used on tumors to detect the present of PD-1 upregulation on T cells or PD-L1 upregulation on tumor or immune cells. The expression of PD-1 on T cells was a poor predictor for response to anti-PD-1 treatments.[178] While PD-L1 expression was significantly correlated with a positive response to immune checkpoint blockade, some patients still have increased survival without expression of PD-L1. [168,172,178,179] Overall, a meta-analysis of 20 trials determined that the objective response rate was higher in patients with PD-L1 expression (34.1%) compared to those without PD-L1 expression (19.9%) in their tumors.[180] When identifying further if the expression of PD-L1 on tumor cells or immune cells was responsible for the

positive patient outcome, the results varied by tumor type. One meta-analyses showed that only PD-L1 expression on tumor cells correlated with an increase in objective response rate.[178] However, in studies investigating individual cancer types, bladder, microsatellite instable colon cancer, and non-bladder genitourinary cancer all had significantly increased positive objective response rates with increased PD-L1 expression on immune cells, but not increased PD-L1 expression on tumor cells.[181–183] Expression of PD-L1 at a primary tumor site does not confer expression of PD-L1 at a metastatic lesion as has been seen in melanoma and renal cell cancer (RCC).[184,185] There are also technical difficulties with the IHC staining of PD-L1. The PD-L1 ligand limits binding regions for IHC antibodies, resulting in heterogeneity of binding efficacy when comparing PD-L1 IHC antibodies.[186,187]

Further investigations have focused on tumor infiltrating lymphocytes, which are increased in the presence of PD-L1 expression.[171] Studies investigating T cell location and recruitment during anti-PD-1 antibody treatments in melanoma patients have identified that the presence $CD8^+$ T cells in the invasive margins and recruitment of $CD8^+$ T cells to the invasive margins and tumor center occur in the patients that respond to treatment but not those that progress.[188] Enzymes that suppress T cell activity have also been investigated, and high levels of IDO expression were correlated with response in melanoma patients.[189] The IFNγ is the cytokine used by cytotoxic T cells when killing tumor cells. Studies have shown that elevated levels of systemic IFNγ and increased levels of IFNγ gene expression are correlated with an increased level of objective response rates compared to patients with low levels of IFNγ or IFNγ gene expression.[190] Given the heterogeneity of carcinogenesis and subsequent immune infiltrates commonly in tumors, we would not predict that a single

type of immune cell or cytokine is the primary predictor for immunotherapy response. Researchers have started using single-cell RNA-sequencing, RNA-Seq, and NanoString pre-treatment tumor data focused on $CD8^+$ T cell characteristics, particularly T cell dysfunction, to more accurately predict patient response to immune checkpoint blockade.[191,192] From TCGA data, high expression of a cluster of Cytotoxic T cell genes including CD8 (*CD8A/CD8B)*, granzyme (*GZMA/GZMB)*, and perforin (*PRF)* were predictive for a positive response to immunotherapy.[191] By investigating many parameters in the tumor immune microenvironment, the overall tumor microenvironment can be used to predict outcomes instead of one immune parameter.

While immune cells are needed to attack tumor cells, tumor-associated antigens that are immunogenic are also required for the T cells to perform cytotoxic functions. Therefore, the tumor mutational burden is also of interest as an immunotherapy response biomarker. A type of colorectal cancer (CRC) has a mismatch-repair (MMR) deficiency, which allows 10 to 100 times more somatic mutations than MMR-proficient tumors due to lack of DNA repair during DNA replication and genetic recombination.[193] MMR-deficient CRC patients have a 40% objective response rate, while MMR-proficient CRC patients have a 0% objective response rate with a progression free survival of 78% and 11% for MMR-deficient and MMR-proficient patients, respectively.[194] While MMR-deficient tumors do have more somatic mutations, studies have also shown they have an increase in tumor-infiltrating lymphocytes, which would further increase the probability of a positive response to immunotherapy.[195] Other types of MMR-deficient cancer patients also have increased objective response rates and progression free survival compared to MMR-proficient patients.[194] Whole-exome sequencing has also identified positive clinical responses to immunotherapy in patients with high mutational burdens.[196] However, similar

to PD-L1 expression, there are patients with high-mutational burdens that do not respond to immune checkpoint blockade.[196,197] A "cutoff" value of tumor mutational burden would also be hard to predict. Different types of cancer have a wide variety of tumor burdens, and even subtypes of organ specific tumors have different tumor burdens based on environmental versus genetic causes. This suggests that each type of tumor may have a different mutational burden cutoff that is predictive of response.

While a tumor may have an increase in mutational burden, immune cells are still needed for antigen presentation, and a highly mutated but "immune cold" tumor with no immune cells may not respond to immunotherapy. Therefore, additional studies are needed to correlate the previously studied biomarkers in combination with mutation burden to determine if patterns can more reliably predict which patients will respond to immune checkpoint blockade. Researchers have started to use predictive models of interactions between immune cells and neoantigens to determine who will respond to immunotherapy.[198,199] The more parameters that can be included from the heterogenous tumor microenvironment, the better predictive models can be in predicting which patients are good candidates for a positive response to immune checkpoint blockade.

After immunotherapy treatment is initiated, it can take up to 6 months to determine if a patient is having clinical benefit from the treatments. Tumors are monitored with serial CT scans, and the response is determined by changes in tumor size. If a tumor enlarges more than 20 percent, the patient is said to have progressive disease per the RECIST criteria.[200] However, sometimes the influx of immune cells causes the increase in tumor size, classifying these cases as tumor-progression. Unfortunately, current clinical imaging methods cannot differentiate pseudo-progression from progression by CT scan. To better visualize cell types changes in the tumor, PET imaging is being used to measure infiltrating

immune cells and their function, which can predict and measure current immunotherapy response. Measuring CD8 T cell infiltration or their granzyme B production are both predictors for positive responses to immune checkpoint blockade. Pre-clinical models of mice with MC38 colon cancer tumors imaged mice with Immuno-PET throughout treatment to identify intratumoral changes in $CD8^+$ and $CD11b^+$ immune cells. Anti-PD-1 responding mice had an increase in $CD8^+$ T cells from the periphery to the center of the tumor as well as an increase in $CD11b^+$ cells to the centers of the tumor.[201] Further analysis of the $CD11b^+$ cells identifying that the responders had an M1-like phenotype of $CD11b^+$ cells, while the non-responders had an M2-like phenotype of $CD11b^+$ cells. These studies show that PET imaging is a successful method of monitoring patients during treatment to determine who will respond to immunotherapy.

Potential Effects of Obesity on Immunotherapy Efficacy

When starting the experiments for the specific aims in 2015, there were no studies investigating how obesity affects immune checkpoint blockade efficacy. During the past five years, there have been several clinical and preclinical studies investigating how the systemic immune dysfunction in obesity results in differences in the tumor immune microenvironment and subsequently how these differences translate to different rates of immunotherapy efficacy between obese and lean subjects. The first article citing a positive effect of obesity on immunotherapy efficacy was by Dr. Jennifer McQuade at MD Anderson. Her study analyzed melanoma patients treated with immunotherapy, detailing that obese men had an increased progression free survival and overall survival compared to lean men.[202] No significant difference in progression free survival or overall survival was found between obese and lean women.[202] Additional studies also found that obese

melanoma patients had increased overall survival with immunotherapy treatment, but their studies showed that this finding was true independent of the patient's sex.[203–205] Both NSCLC and RCC also have increased overall survival in obese patients treated with immunotherapy compared to patients with a normal BMI.[206,207] In the RCC study, the authors correlated survival with a systemic inflammation index (SII) that they had previously used to correlate prognosis for RCC, hepatocellular cancer, and prostate cancer.[208–210]

$$SII = Neutrophils\ x\ \frac{Platelets}{Lymphocytes}$$

The SII is determined with pre-treatment neutrophil, platelet, and lymphocyte counts. Patient's with a low systemic inflammation index (SII < 1,375) had significantly increased overall survival compared to patients with a high SII (SII ≥ 1,375).[206] An additional prospective study of clear cell RCC also found that overall survival was longer in obese patients treated with immunotherapy.[211] There was no significant difference in immune cell infiltration between obese and lean patients, but obese patients did have higher angiogenic scores on gene-set enrichment analyses and increase peritumoral adipose tissue inflammation compared to patients with normal weights.[211] This data further supports how adipose tissue adjacent to or encapsulating tumors can effect treatment outcomes in obese patients.

Body mass index is determined by a patient's height (m) and weight (kg), and it does not include information on a patient's adipose tissue or muscle mass.

$$BMI = \frac{Weight\ (kg)}{Height\ (m)^2}$$

To tease apart the potential effects of adipose tissue versus muscle in improved overall survival, researchers correlated both BMI and serum creatinine concentrations (an indicator of skeletal muscle mass) with overall survival for melanoma patients treated with immune checkpoint blockade.[212] BMI was divided into normal BMI (18.5< BMI<25), overweight/obese class I BMI (25 BMI < 35), or obese class II/III (BMI > 35), while creatinine was divided into high creatinine ≥ 0.9 mg/dL or low creatinine < 0.9 mg/dL. The study showed that overweight/obese class I patients with a high creatinine level had the longest overall survival.[212] Another study investigated only skeletal muscle content with immunotherapy-treated patient outcomes. The skeletal muscle index (SMI) and skeletal muscle density (SMD), provided quantitative and qualitative data about the skeletal muscle, respectively.[213] The SMD was calculated from adipose tissue distribution around the muscle and muscle atrophy from inactivity. Patients with a high SMI had increased progression free survival and overall survival compared to patients with a low SMI.[213] However, there was no significant difference in objective response rate to the immunotherapy treatment between high and low SMI patients. Also, there were no significant increases in progression free survival or overall survival for patients with a low SMD versus high SMD.[213] Together, these data suggest that sarcopenia is an additional prognostic factor that needs to be taken into consideration when investigating obesity's effects on immunotherapy efficacy.

While numerous studies have investigated clinical data to identify the effects of obesity on immunotherapy efficacy, there have been limited preclinical studies to identify the mechanism of these effects. The first published preclinical study from Dr. William Murphy's group in 2019 detailed that T cells from non-tumor bearing obese mice, monkeys, and humans had increased expression of PD-1 and decreased proliferation.[214]

The T cells from obese mice also had decreased IFNγ and TNFα production, suggesting decreased function in non-tumor bearing mice. Obese C57BL/6 mice with B16 melanoma tumors grew larger tumors than lean mice, and the obese mice had a decreased frequency of proliferating $CD8^+$ TILs and an increased frequency of $CD8^+$ TILs expressing PD-1, Tim3, and LAG3 checkpoint blockades compared to lean mouse $CD8^+$ TILs.[214] The B16 melanoma cell line does not classically respond to immune checkpoint blockade. However, when anti-PD-1 treatments were given to B16 tumor-bearing obese and lean mice, the tumor volumes of obese mice decreased to that of the lean mouse tumors, and there was no significant difference between the size of lean mice tumors treated with control IgG and anti-PD-1 antibody.[214] Obese mice treated with immunotherapy had a significantly increased frequency of $CD8^+$ TILs in the immune cell fraction and significantly decreased metastatic burden compared to lean mice.[214] There was a positive correlation between leptin serum levels in peripheral human blood with the frequency of $PD1^+$ $CD8^+$ T cells. Given this, they identified that while *db/db* mice who lack the leptin receptor become obese, they have significantly decreased frequencies of $PD1^+$ $CD8^+$ T cells in the spleen and liver compared to wild type DIO C57BL/6 mice. Additionally, when stimulating T cells with anti-CD3 *in vitro*, the addition of exogenous recombinant leptin protein increased the frequencies of $PD1^+$ $CD8^+$ T cells compared to T cells stimulated with only anti-CD3. These studies effectively described obesity's effects on T cells, tumor growth, and anti-PD-1 treatment efficacy, suggesting that leptin plays a role in increased checkpoint expression on T cells. However, more studies are needed to uncover how additional immune cells, such as macrophages or myeloid derived suppressor cells, are affected by obesity and how they contribute to immunotherapy efficacy.

An immune response is needed to achieve an objective response to immune checkpoint blockade (ICB), but over activation of the immune system can lead to immune-related adverse events (irAEs). An irAE can range from a Grade 1 case of inflammation such as mild colitis and dermatitis that allow continued use of ICBs, to moderate cases that require discontinuing ICB treatment such as hepatitis induced cirrhosis or skin blistering, to life-threatening Grade 4 cases including myocarditis and encephalitis.[215] Systemic IL-2 treatment is an older type of cancer immunotherapy for melanoma and renal cell cancer that can also generate immune-related toxicities. The IL-2 treatments induce T cell activation and proliferation, subsequently increasing associated cytokines. This mechanism creates a cytokine storm, similar to sepsis, that helps the T cells attack the cancer cells. However, the balance between immune system activation and life-threatening cytokine storm can be difficult to achieve, and the IL-2 toxicities can result in patient death. Dr. William Murphy investigated the effects of age and obesity on IL-2 toxicities and discovered that older and obese mice developed lethal cytokine storms after IL-2 treatments.[216,217] In both the aged and obese mouse models, macrophages had a greater M1 phenotype with increased TNF and IL-6 production compared to young or lean mice, respectively.[216,217] By blocking macrophages with clodronate in either model, the lethal toxicities with IL-2 treatment did not occur.[216,217] Similarly, when investigating irAEs in patients treated with immune checkpoint blockade, both obese and elderly patients had increased risks of developing irAEs compared to young and lean patients.[218–220] To increase the translational potential in preclinical models of immunotherapy, identifying immune toxicities and potential mechanisms of these toxicities is important for patient safety and improved patient care.

Leptin Effects on Immune Cells

Adipose tissue was originally viewed as a fat storage organ, but after leptin's discovery in 1994, scientists began to understand that the organ had important roles in the endocrine system. Adipocytes are the primary producer of leptin. Leptin's main endocrine function is energy homeostasis, and subsequently leptin levels correlate with insulin levels and the amount of energy stored in adipose tissue (body adipose mass).[221] As its primary function, leptin plays a role in neuroendocrine signaling to induce satiety and increase energy consumption.[222] In the hypothalamus, leptin inactivates adenosine 5'-monophosphate kinase (AMPK), which increases acetyl-CoA carboxylase (ACC) activity and decreases food intake.[223] Meanwhile, in skeletal muscle, leptin deactivates AMPK, increasing fatty acid oxidation.[223] Overall, leptin plays a direct role in managing food intake as well as inducing fatty acid metabolism.

While leptin's role was initially thought to be purely endocrine, increasing evidence identifies important roles for leptin in immune system regulation.[221] When the body has sufficient energy via fat storage, leptin signals to the hypothalamus that enough storage is present for the activation of immune cells.[224] In the hypothalamus, leptin binds to leptin's functional receptor (ObR). While ObRs are encoded by the diabetes (db) gene, ObRs are part of the class I cytokine receptor superfamily, including the IL-6 receptor. There are six different isoforms of the receptor in mice: four short isoforms ObR_a (leptin transporter across blood-brain barrier and leptin degrader), ObR_c, ObR_d, and ObR_f, one long isoform, ObR_b, and a secreted isoform, ObR_e, that is a plasma membrane due to its lack of intracellular and transmembrane domain.[224] The ObR_b is expressed on the majority immune cells including T cells, macrophages, natural killer cells, dendritic cells, and neutrophils.[225–228] Additionally, leptin's structure is part of the type I cytokine family,

which is similar to that of the IL-6 cytokine.[229] When leptin binds to the ObR_b receptor on immune cells, the long domain has motifs that allow for activation of the Janus family 2 (JAK2)/STAT3 pathway, which alters gene transcription and gradually increases inflammation.[230–233] Leptin also works on alternative pathways through JAK2, such as the extracellular signal-regulated kinase (ERK) – 1/2 and the phosphatidylinositol-3-kinase (PI3K) pathways, which can be activated by ObR_a or ObR_b leptin receptors.[224] In ERK signaling, phosphorylated Tyrosine[985] of JAK2 and phosphorylated JAK2 itself will activate and phosphorylate *src* homology 2-containing tyrosine phosphatase (SHP-2). Then, SHP-2 associates with the growth factor receptor binding 2 (Grb-2) protein and activates the ERK/MAPK pathway, which is leptin's main mechanism of regulatory T cell regulation.[234] JAK2 activation also phosphorylates insulin receptor substrate 1 (IRS1), which activates phosphatidyl 3'kinase (PI3K), leading to cellular proliferation.[235] Leptin also activates the mitogen-activated protein kinase (MAPK), supporting additional proliferation, differentiation, and cell growth.[231,232] Activation of the PI3K pathway does not require phosphorylation of a tyrosine on ObR_b. Instead the autophosphorylation of JAK2 recruits and phosphorylates insulin receptor substrate 1 and 2 (IRS 1/2), which recruit PI3K, whose downstream effects contribute to acute inflammation including Akt and mTor.

Leptin's effects on the immune system are both direct and indirect and effect both innate and adaptive immunity. Children with congenital leptin deficiency have an increased risk of infection-related death, and mice without leptin or ObR have defects in both innate and adaptive immunity.[236–238] In the innate immune system, leptin acts directly on immune cells to promote inflammation. Leptin polarizes macrophages towards an M1 phenotype with increased phagocytic function and pro-inflammatory cytokine secretion of TNFα, IL-

6, and IL-12 (**Figure 1.5**).[239] Eicosanoid, leukotriene, nitric oxide synthesis, and IFNγ-induced expression of nitric oxide synthase are also promoted by leptin, furthering an M1 macrophage phenotype.[227,240,241] *Ob/ob* mice are deficient in the ob gene that is needed to produce leptin, and *db/db* mice are deficient in the leptin receptor. Both *ob/*ob and *db/*db mice have decreased macrophage phagocytosis and decreased expression of pro-inflammatory cytokines, whereas mice provided exogenous leptin have macrophages with increased phagocytosis and production of pro-inflammatory cytokines.[242] In the circulation, leptin also stimulates proliferation of monocytes and upregulates the activation markers CD25 (IL-2 receptor alpha chain), CD38 (cyclic ADP ribose hydrolase), CD69 (C-Type lectin), CD71 (transferrin receptor), HLA-DR (MHC Class II receptor), CD11b, and CD11c.[227] Leptin's activation of protein kinase C and nitric-oxide dependent pathways increase growth hormone production by peripheral-blood mononuclear cells.[243] In natural killer cells, leptin activates STAT3 and up-regulates perforin and IL-2 production to increase natural killer cell differentiation, proliferation, activation, and cytoxicity.[226,244] Leptin induces neutrophil chemotaxis and their release of reactive oxygen species.[228,245] When leptin binds to the ObR_b receptor on dendritic cells, there is a decrease in macrophage inflammatory protein-1-α production but an increase in IL-8, IL-12, IL-6, and TNFα production by dendritic cells.[246] Leptin also increases dendritic cell survival and their surface marker expression of CD1a, CD80 (B7), CD83, and CD86 (B7-2), which play a role in antigen presentation and encourages Th1 priming.[246] *Db/db* mice have a Th2 T cell and cytokine profile, decreased co-stimulation between DCs and T cells, decreased PI3K/Akt pathway activity, and decreased STAT3 activity.[246] In neutrophils, leptin increases chemotaxis, survival, and secretion of oxygen radicals directly through the leptin

receptor and indirectly by increasing TNFα levels.[221,247] Eosinophils also have leptin receptors, where leptin activates eosinophils and inhibits apoptosis, as well as stimulating chemotaxis, cytokine release, and adhesion molecule expression.[248,249] In the innate immune cells, leptin creates an influx of Th1 and M1 pro-inflammatory innate cells, which is physiologically useful in the setting of infection.[223]

While leptin's effects on innate immunity are largely direct, leptin alone cannot effect adaptive immunity.[221] When leptin is co-administered with immunostimulants, an upregulation of both early and late activation markers are seen in both $CD4^+$ and $CD8^+$ T cells including CD69, CD25, and CD71.[250] When stimulating T cells *in* vitro with anti-CD3, T cells receiving leptin had increased production of IFNγ compared to control T cells (**Figure 1.5**).[251] Similar to the macrophages, leptin stimulates activation of the pro-inflammatory Th1 T cell, increases cytokine production, and upregulates the adhesion molecules membrane glycoprotein Ia-IIA (VLA-2) and intracellular adhesion molecule 1 (ICAM-1).[225,250,252] Additionally, leptin receptor signaling is necessary for Th17 cell differentiation.[253] Leptin supports Th1 and Th17 cell proliferation and survival through an autocrine loop of leptin secretion by Th1/Th17 T cells as well as upregulation of Bcl and Tbet and STAT3 activation, respectively.[225,251,254–256] The T cells in *ob/ob* and *db/db* mice have decreased IL-2 and IFNγ production and a subsequent decrease in T cell mediated immunity, but these deficits are restored with chronic exogenous leptin treatments.[225,257] Through increasing pro-inflammatory cytokines, leptin is a negative regulator of T regulatory cell proliferation. In *in vitro* T cell stimulation with anti-CD3, anti-CD28, and leptin neutralization or in an *in vivo ob/ob* mouse model, T regulatory cell proliferation is increased via the mTor pathway as compared to control *in vitro* stimulation conditions or

wild type mice, respectively. In the thymus, leptin prevents glucocorticoid-induced apoptosis of thymocytes and increases thymus cellularity, further increasing T cell proliferation and survival.[257] The effects of leptin on B cells are not as well understood as leptin's effects on T cells. Given B cells role in antigen presentation to T cells and secretion of pathogenic antibodies in adipose tissue, particularly in obesity, there is a presumed association of leptin increasing B cell intrinsic inflammation and contributing to proinflammatory T cell function.[44,258] Both in innate and adaptive immunity, leptin is a powerful adipokine that increases inflammation in almost all immune cells.

Leptin's increase in inflammation is also associated with a variety of inflammatory disorders. High serum leptin levels correlate with autoimmune disease activity in patients, and decreasing leptin levels via calorie restriction correlates with decreased autoimmune disease severity.[259–264] Specifically, patients with rheumatoid arthritis that fast have a decrease in circulating leptin, a subsequent decrease in CD4 lymphocyte activation, and an increase in IL-4 levels due to increased Th2 T cell proliferation.[265] Animal models of colitis have elevated leptin levels, and these mice develop anorexia.[266] In humans with ulcerative colitis, serum leptin levels are elevated in acute flares, during which the inflamed colonic epithelial cells secrete leptin, activating NF-κb.[267] Both macrophages and T cells also produce leptin in models of autoimmune encephalomyelitis, identifying that leptin increases inflammation through not only an endocrine fashion, but also an auto or paracrine mechanism.[268] Meanwhile, leptin-deficient mice are resistant to the induction of autoimmune diseases such as colitis, autoimmune encephalomyelitis, type I diabetes, and hepatitis.[269] These studies identify that leptin is an important component to the development of acute flares in autoimmune disease.

Leptin Directly Increases Pro-Inflammatory Immune Cells

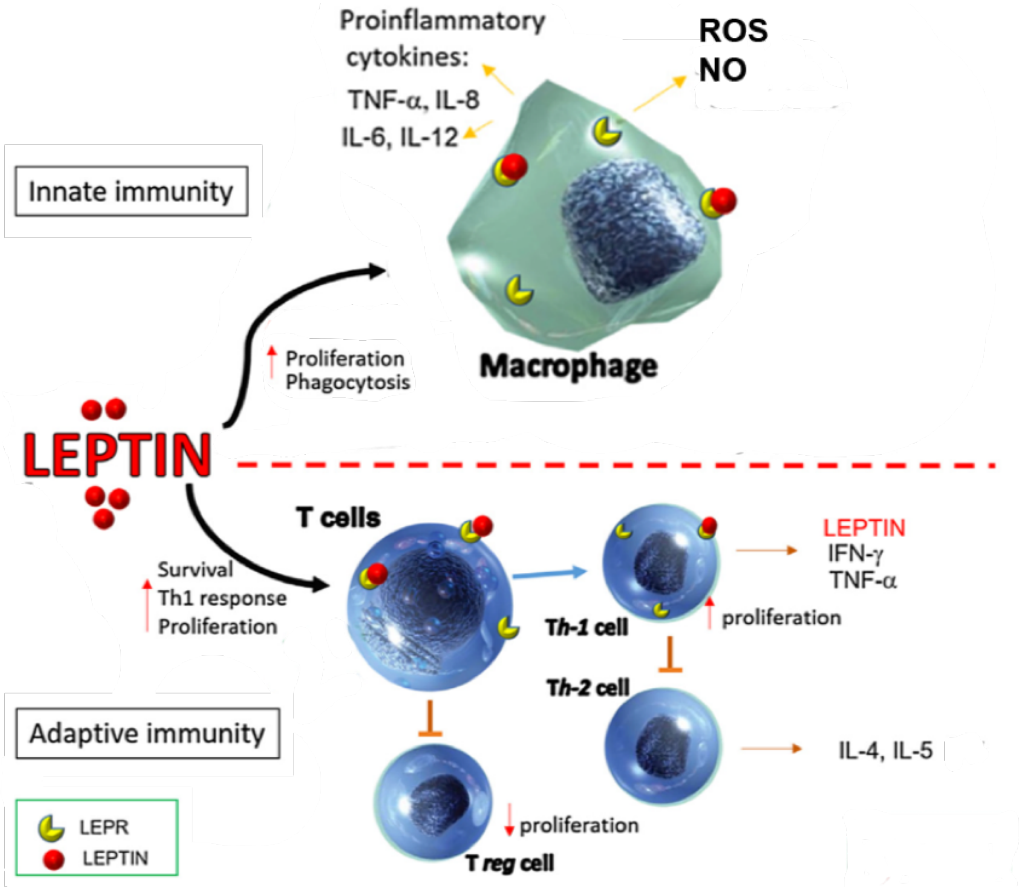

Figure 1.5 Leptin acts directly through leptin receptors on immune cells to increase inflammation. Leptin increases M1 polarization of macrophages through the JAK/STAT pathway to increase phagocytosis and secretion of pro-inflammatory cytokines, reactive oxygen species (ROS), and nitrogen oxide (NO). Through antigen presentation and binding the leptin receptor on T cells, the T_H1 T cell survival and proliferation increases. Additionally, proliferation of the T regulatory cells decreases, and the T_H2 and T regulatory cell functions are inhibited. The T_H1 cells secrete IFNγ, which further supports the M1 macrophage phenotype.[246]

Due to its structure, leptin can also act as a pro-inflammatory cytokine, independent of its direct effects or immune cells. Inflammatory cytokines that appear during acute infection and sepsis, such as IL-1, IL-6, LPS, and TNFα, can increase leptin mRNA expression and stimulate leptin release from adipose tissue.[270] Murine models of acute inflammation are associated with increased serum leptin levels.[269] While acute pro-inflammatory cytokines lead to a rise in leptin levels and its associated pro-inflammatory

effects, leptin does not function the same in chronic inflammatory settings.[223]

Prolonged and continuous adipose tissue accumulation results in obesity, where obese subjects have high leptin levels that correlate with and may be causative of high insulin levels and insulin resistance.[246,271] While leptin induces a proinflammatory immune cell state, obese subjects, despite an elevated leptin levels, have an increased risk of infection.[221] Obese rats have elevated leptin and decreased mRNA of the ObR_b receptor compared to lean mice, suggesting a state of leptin resistance.[272] The leptin resistance is likely due to expression of suppressor of cytokine signaling 3 (SOCS-3) via STAT3 activation.[273,274] T cells from obese mice have decreased proliferative rates, despite leptin's induction of T cell proliferation, but these T cell abnormalities are reversed with calorie restriction by decreasing leptin levels.[272,275] Leptin levels are not only correlated with energy intake but also TNFα levels, which are elevated in obesity.[275,276] Using a leptin-deficient mouse model that over expressed adiponectin, the diet-induced obese mice had increased subcutaneous fat without an increase in adipose tissue macrophages, no increase in visceral adipose tissue, and no development of metabolic syndrome due to lack of pro-inflammatory changes typically seen in obesity.[277] Indeed, leptin deficient mice and acute starvation induces immunosuppression, where the immune response can be stimulated by exogenous leptin.[225,278] Of note, both *ob/ob* and *db/db* leptin-deficient mice have elevated cortisol and hyperglycemia but the immune profile fits that of a starvation model.[257] When restricting food in *ob/ob* and *db/db* mice, glucose and cortisol levels decrease but immunosuppression persists.[225] As insulin resistance progresses in diabetes patients, some patients require exogenous insulin to maintain glucose homeostasis. However, if leptin is provided to diet-induced obese subjects, the exogenous leptin did not induce weight loss nor did it change immune cell proliferation, activation or polarization.[279,280] These studies

suggest that the exogenous leptin cannot overcome the resistance developed in obesity.

The acute effects of leptin on immune cells have tremendous potential for enhancing immunotherapy efficacy. While immunotherapy can decrease anti-PD-1 or anti-CTLA-4 expression, the addition of leptin would further support M1 macrophage polarization, cytotoxic T cell function by decreasing T regulatory cells, increasing IFNγ production, and promoting a Th1 CD4 T cell phenotype (**Figure 1.6**).

The Role of Tumor-Associated Macrophages in Immune Checkpoint Blockade Efficacy and Potential for Leptin to Enhance Immunotherapy Efficacy

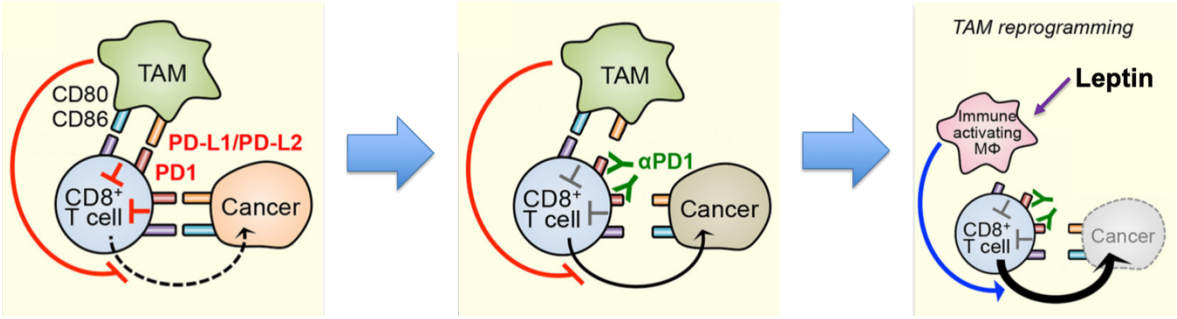

Figure 1.6. Both cancer cells and tumor-associated macrophages (TAMs) express PD-L1, which inhibit $CD8^+$ T cells through the PD-1/PD-L1 axis. However, TAMs also secrete cytokines, such as IL-10, that inhibit $CD8^+$ T cell function too. Anti-PD-1 antibodies block the PD-1/PD-L1 axis and rescue some $CD8^+$ T cell effector function, but the TAMs will still inhibit T cells. Full effector function can be achieved by repolarizing the TAMs to an M1-like macrophage. Leptin increases M1 polarization of macrophages and has the potential to increase immune checkpoint blockade efficacy through macrophage repolarization.[281]

Radiation and the Abscopal Effect

Shortly after the discovery of x-rays in 1895, scientists began using x-rays and radiation experimentally in the late 19th century and clinically early in the 20th century, without understanding x-rays effects on tissues.[282] After half a century of clinical use, scientists recognized that the primary mechanism for radiation treatment of tumors was DNA damage via high-energy deposition of x-rays.[283,284] The cancer cells that mutated, inducing rapid proliferation, were no longer able to repair themselves after DNA damage,

while the normal healthy cells still possessed DNA repair mechanisms. Consequently, the cancer cells either underwent apoptosis or growth arrest, resulting in cancer cell death. When the DNA damage mechanism was uncovered, the abscopal effect was identified simultaneously by R.H. Mole, who coined the term abscopal as "action at a distance from the irradiated volume but within the same organism".[285] In Mole's experiments, mice had two tumors, where one tumor received radiation and the either did not. Despite only treating one tumor, Mole observed a reduction in tumor volume in both tumors.[286] While the primary radiation treatment mechanism through DNA damage has been thoroughly studied and is now well understood, clinicians are still unable to predict when an abscopal effect will occur in patients, and it has overall been a rare occurrence in patient care.

Despite the minimal predictability for inducing systemic anti-tumor immunity through the abscopal effect, the immune system is critical in initiating an abscopal effect.[285] With radiation treatment, the apoptosis and necrosis of tumor cells can release tumor antigens creating an immunogenic cell death and release of damage-associated molecular patterns (DAMPs).[287] The DAMPs initiate dendritic cell recruitment to the tumor site, boosting antigen presentation to T cells.[288,289] The dying tumor cells release DNA, which further improves antigen presentation through activation of the stimulator of interferon gene (STING) pathway and IFNγ in dendritic cells.[290] Radiation damage induces calreticulin transportation to the surface of dying cancer cells, which serves as an "eat me" signal for dendritic cells.[291] Damaged cells that survive radiation have increased expression of intercellular adhesion molecule 1, death receptor Fas, and MHC-I, which increase tumor antigen recognition by antigen presenting cells.[292] The tumor cell death also induces anti-tumor associated cytokines, increasing MHC expression and permeability of blood vessels, which allows more T cell and dendritic cell entrance into the tumor tissue,

further promoting an anti-tumor response.[289,293,294] After low-dose radiation, monocytes are recruited to the tumor site, and their engulfment of dying tumor cells promotes a M1 phenotype, resulting in increased T cell infiltration.[295] While the initial M1 polarization supports anti-tumor activity, sustained inflammation from M1 macrophages increases the risk of carcinogenesis in nearby tissues.[296,297] In sporadic radiation-induced abscopal effects, the majority of cases occur in immunogenic tumors such as renal cell carcinoma, melanoma, and hepatocellular carcinoma, where half of these cases radiation therapy delivered to the primary tumor and the other half had radiation therapy delivered to metastatic sites.[298,299] Given these mechanisms, murine experiments providing radiation treatment in combination with either dendritic cell growth factors, TLR7/Imiquimod to enhance antigen presentation, or IL2 to increase T cell proliferation and activation induced greater abscopal effects and decreased metastasis rates.[300–304] While the induced inflammation is positive for an abscopal effect, the inflammation can also effect normal tissues leading to a range of mild side effects (nausea and fatigue) to severe side effects (pneumonitis).[305,306] Similar to the abscopal effect, the severe side effects cannot be predicted yet by patient demographics. These approaches have been promising in mouse models, but limited success has occurred in clinical trials. This may be due to the immunosuppressive effects induced by radiation treatment including increasing TGFβ, MDSC recruitment, and T regulatory cell recruitment.[307,308] New treatments to decrease immunosuppression, increase tumor antigen recognition, and increase anti-tumor immune cell activity need to be explored further to induce a reproducible abscopal effect when co-treated with radiation. While initial abscopal effects were observed in conventional radiation therapy (more than 5 treatments, <6 Gray (Gy) each). As clinicians have increased implementation of stereotactic body radiotherapy (less than 5 treatments >6 Gy

per treatment), cases of abscopal regression have been recorded in renal cell cancer and lung cancer.[309–311] The ability to induce an abscopal effect would be clinically powerful to improve patient quality of life through reduction of tumor burden. More importantly, the ability to induce an abscopal effect that is permanent is the goal as the median duration for an abscopal response is 21 months (range 3 – 54 months).[299] Based on previous studies investigating radiation and the abscopal effect, monitoring cytokine production and antigen presentation are parameters that could help determine, which patients may have an abscopal effect or potentially toxicities, like pneumonitis.[299] Other tumor intrinsic components that may influence abscopal effects are genomic instability and mutation burden, hypoxia, tumor cell metabolism, proliferation rate, and intrinsic tumor cell radiosensitivity.[299]

Radiation and Immunotherapy

While the abscopal effect seen with radiation treatment alone is unpredictable and rare, the addition of immunotherapy to treatment regiments may make inducing abscopal effects a therapeutic goal. Shortly after the discovery of immune checkpoint antibodies, preclinical studies investigating the effects of immunotherapy on enhancing radiation treatment were initiated. When combining anti-CTLA-4 with radiation in immunogenic MC38 colon cancer or the poorly immunogenic 4T1 orthotopic murine breast cancer model, the mice had increased survival, decreased rate of tumor growth, and decreased metastases rate in the 4T1 model, and these findings were not seen in anti-CTLA-4 or radiation treatment alone.[312,313] Similarly, co-treatments with radiation and anti-PD-1 antibodies have decreased tumor growth and increased survival has in murine models of melanoma and breast cancer.[314] Some murine studies have demonstrated that tumor

resistance can occur after co-treatment of anti-CTLA-4 and radiation, when an upregulation of anti-PD-L1 on melanoma cells occurred in the murine study.[315] Early into the implementation of immune checkpoint blockade as a cancer treatment, a metastatic melanoma patient experienced an abscopal effect after receiving anti-CTLA-4 and radiation treatment.[316] The patient received palliative radiation for a paraspinal mass concurrently with anti-CTLA-4 antibodies. Not only did the paraspinal mass regress, but a right hilar lymph node and spleen metastases regressed four months after radiation treatment. In a phase I/II clinical trial, 34 castration-resistant prostate cancer patients with bone metastases received radiation to the metastases and anti-CTLA-4 antibodies. Only one patient had a complete response with metastasis regression, and six patients had stable disease with greater than fifty percent decrease in prostate-specific antigen.[317] This small success rate further supports the need to better understand how to optimize the combination of immunotherapy and radiation treatment to increase the anti-tumor immune response and odds of inducing an abscopal effect.[287]

Specific Aims

Obesity is a risk factor for at least 13 different types of cancer, and in the United States 34.9% of adults are obese. Along with an increased risk of developing cancer, obese patients also have worse outcomes and response to surgery, chemotherapy, and radiation therapy treatments. There have been no studies investigating how well the newest cancer treatment method, immunotherapy, works in obese patients. Given that only a small fraction of patients has a significant clinical response to immune-checkpoint blockade treatments, there is good evidence that immunotherapy treatments will be ineffective in the obese population. Obesity, promotes an increase in adipose tissue macrophages, which tend to have a pro-inflammatory phenotype. While this inflammation can promote cancer cell survival and tumor progression, these chronic immune changes also impact and suppress adaptive, and potentially, anti-tumor immunity. As time progresses, studies have shown that the adipose tissue macrophages share M1 and M2 characteristics. One theory is that the tumor microenvironment shifts the macrophages from the M1 phenotype towards a tumor-associated macrophage, M2 phenotype. Obese individuals also have a decreased humoral immune response due to a multitude of factors including an altered lymphocyte profile, decreased lymphocyte functionality, dysregulated cytokines, and decreased numbers of dendritic cells, natural killer cells, cytotoxic $CD8^+$ T cells, and macrophages. It is not clear how these immunological impairments caused by obesity will affect tumor associated inflammation and response to immunomodulatory therapies. It is hypothesized that a combination of the long-term high levels of circulating pro-inflammatory cytokines in conjunction with the changes in insulin signaling and elevated glucose levels are the main players causing immune dysfunction in obesity, but no mechanistic studies have been

conducted to date. To solve the problem of poor efficacy with immunotherapy, additional therapy can be used to create a stronger anti-tumor immune microenvironment. Leptin is a hormone that is elevated in obesity, and previous studies have shown that leptin polarizes macrophages to an "M1" anti-tumor phenotype. If immunotherapy efficacy is enhanced in obesity, the effects of leptin on the tumor immune microenvironment and anti-PD-1 treatment response need to be further studied. *The overall goals of this thesis are 1) to understand the effects of obesity on immunotherapy efficacy, 2) to determine if immunotherapy efficacy can be improved with the leptin hormone, 3) to determine if immunotherapy can enhance radiotherapy***.** I therefore propose three independent but complementary aims that will identify how obesity changes affects immunotherapy efficacy, determine if immunotherapy efficacy can be improved through exogenous leptin treatments, and investigate if radiation treatments can enhance immunotherapy treatments and induce an abscopal effect.

Specific Aim 1: To determine the effects of obesity on anti-PD-1 treatment efficacy. Experiments in this aim will test the hypothesis that *lean mice with subcutaneous MC38 colon cancer tumors will have different efficacy rates with anti-PD-1 treatment than obese mice with MC38 colon cancer tumors.* In ***subaim 1.1****,* I will characterize the tumor progression and baseline immune cell profile of diet-induced obese and normal weight tumor-bearing mice using flow cytometry, histology, and cytokine analysis. In ***subaim 1.2***, I will measure tumor progression and treat obese and normal weight tumor-bearing mice with anti-PD-1 antibodies to test the ability of this immune checkpoint therapy to alter tumor immune cell infiltrates and inflammation using flow cytometry and histology.

Specific Aim 2: To explore the role of leptin on the tumor microenvironment and its potential for enhanced immunotherapy efficacy.

Experiments in this aim will test the hypothesis that *leptin will repolarize tumor-associated macrophages to a pro-inflammatory "M1" phenotype, improving immune-checkpoint blockade efficacy by creating a stronger anti-tumor immune microenvironment.* In ***subaim 2.1***, I will test how leptin treatments of lean animals with subcutaneous MC38 colon cancer tumors affect tumor growth and the tumor immune microenvironment. In ***subaim 2.2***, I will test if the co-treatment of leptin and anti-PD-1 produces the greatest tumor treatment efficacy in lean mice. For Aim 2 subaims, I will measure tumor progression and conduct immune analysis as in Aim 1.

Specific Aim 3: To determine if co-treatments with radiation and immune checkpoint blockade improve overall survival and induction of the abscopal effect.

Experiments in this aim will test the hypothesis that *combining immune checkpoint blockade treatment with radiation will increase the abscopal effects and enhance the anti-tumor immune response more than either treatment alone*. Myc-CaP, castration-resistant prostate cancer cell, will be injected into the leg and flank of mice. In ***subaim 3.1***, I will test if co-treatments with anti-PD-1 or anti-PD-L1 with radiation to the leg tumors improve overall survival and increase immune cell infiltrate compared to control, anti-PD-1, or anti-PD-L1 treatments alone. In ***subaim 3.2*****,** I will test if $CD8^+$ T cells are required for the enhanced immune response induced by radiation and immune checkpoint blockade by simultaneously treating mice with anti-CD8a antibodies.

II. THE EFFECTS OF OBESITY ON IMMUNOTHERAPY EFFICACY

Abstract

Immunotherapy is life-saving for some cancer patients and while obesity can promote cancer, it may also increase immunotherapy efficacy. Mechanisms of this effect have been unclear, although obesity can promote an inflammatory state to modify tumor infiltrating lymphocytes and tumor associated macrophage (TAM) populations in tumors. To identify mechanisms by which obesity affects anti-tumor immunity, diet-induced obese and lean mice received subcutaneous MC38-CEA1 colon cancer tumors. Obesity increased tumor growth rate and tumor burden. Fewer immune cells were present in tumors from obese mice, and the T cells had an activated an exhausted phenotype. Macrophages in obese animals had decreased expression of iNOS2 and MHCII, supporting a weaker "M1" anti-tumor phenotype compared to lean mice. When treated with anti-PD-1 antibodies, however, obese mice had a larger decrease in tumor burden than lean mice. While PD-1 blockade repolarized the macrophages to an M1-phentoype in obese mice, this did not occur in the lean mice. These data demonstrate that obesity has dual effects in cancer, promoting tumor cell growth while also priming anti-tumor immunity through elevated levels of leptin that can reprogram inflammatory macrophages.

Introduction

Obesity is a risk factor for at least 13 different types of cancer, and in the United States 34.9% of adults are obese.[7] It is estimated that obesity is the cause of 14% and 20% of cancer deaths in men and women, respectively.[10,11] Intestinal cancers, such as esophageal, stomach and colorectal have the highest relative risk with obesity.[10] Along with an increased risk of developing cancer, obese patients also have worse outcomes and response to surgery, chemotherapy, and radiation therapy treatments.[16] Early published studies investigating how well immunotherapy works in obese patient and mouse models have indicated that obesity can improve immunotherapy efficacy, but the complete mechanism for this is not yet understood.

In obese patients, chronic lipid and nutrient overload can lead to increased inflammation in adipose tissue that promotes the constellation of pathologies termed the metabolic syndrome.[318] Even prior to overt conditions of the metabolic syndrome such as type II diabetes or cardiovascular disease, immunity and immune responses are altered and impaired.[319] Adipocytes that accumulate excessive fat begin to secrete inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemotactic protein (MCP-1); TNF-α and IL-6 cytokines are known to be elevated in colon cancer.[320–323] Adipose-secreted cytokines (adipokines) leptin and adiponectin are also altered in obesity. High levels of leptin are associated with increased colon cancer risk, while high levels of adiponectin correlate with a decreased risk of colon cancer.[324–326] Leptin and MHCII expression by adipocytes and myeloid cells skew and activate T_H1 cells, and the T_H1 and $CD8^+$ T cells secrete elevated levels of interferon-γ (IFN-γ).[35] Obesity also triggers intracellular pathways that upregulate cyclo-oxygenase-2 (COX2), signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) pathways, which

increase inflammation, cellular proliferation, and anti-apoptotic proteins.[119,327–331] As metabolic syndrome develops, the concomitant high glucose concentrations further promote a pro-tumor microenvironment by activating Hif1α, and hyperinsulinemia further promotes cell growth and anti-apoptotic proteins.[332] Additionally, excess inflammation is involved in the epithelial-to-mesenchymal transition, which can increase cells metastatic potential and genomic instability, resulting in increased DNA damage and mutation load.[331]

Macrophages in normal tissues are quiescent or of the alternatively activated (AAM/M2) phenotype. In obesity, macrophages are recruited through MCP-1 and TNF-α, and the elevated IFN-γ in adipose tissue promotes a shift towards a classically activated/inflammatory (CAM/M1) phenotype and MHCII expression in the adipose tissue macrophages. While inflammation can promote cancer cell survival and tumor progression, these chronic immune changes also impact and suppress adaptive, and potentially, anti-tumor immunity. Adipose tissue macrophages share M1 and M2 characteristics.[333] However, the phenotype of tumor-associated macrophages (TAMs) is not the same as that of the adipose tissue.[334] In a tumor, the M1 macrophage has anti-tumor properties via its production of pro-inflammatory cytokines and nitric oxide synthase, while the M2 macrophage has immunosuppressive properties due to its tissue repair functions and anti-inflammatory cytokines.[140]

Immunotherapy treatments for cancer aim to treat and cure cancer patients through modulation of a patient's own immune cells. One of these treatment options uses antibodies that bind to the programmed death-1 (PD-1) receptor. The use of an antibody to PD-1 recovers the "exhausted" T-cell function and leads to subsequent tumor cell death.[335] This treatment is life-saving for some patients, but clinical trials have demonstrated limited efficacy of anti-PD-1 (αPD-1) with approximately one-quarter to one-third of patients

showing a partial or complete response, and clinicians still cannot completely predict which patients will respond.[336] While many studies have focused on tumor-associated factors, such as the T cell infiltration, PD-1 or PD-L1 expression, and mutation burden, there have been fewer studies focusing on patient-associated factors such as age, smoking habits, diet composition, and patient body mass index (BMI). Studies investigating the effects of obesity on immunotherapy efficacy have largely focused on T cell phenotyping, identifying that obesity induces increased immune checkpoint blockade markers in T cells.[214] However, the mechanism of how obesity affects immunotherapy remains unknown, especially with respect to the roles of macrophages and other non-T immune cells.

TAMs usually make up the greatest portion of immune cells in tumors, and these macrophages typically have an M2 phenotype that supports tumor growth and inhibits anti-tumor immune cells.[127–132] Monocytes are recruited to tumors through the chemokines CCL2, CCL5, vascular endothelial growth factor (VEGF), TGFβ, and colony stimulating factors (GM-CSF and M-CSF).[128,155,337–339] The recruited monocytes then mature into M2-polarized macrophages due to the intratumoral cytokines, M-CSF, PGE_2, TGFβ, IL-6, and IL-10.[340,341] TAMs also promote tumor progression and invasion by secreting matrix metalloproteinases and cathepsins that degrade extracellular matrix (ECM) proteins, while TAMs also promote angiogenesis.[342] Blood vessels needed for tumor progression are supported through TAM production of TGFβ and VEGF.[127,343,344] Due to these pro-tumor functions, the majority of cancers have an association between high TAM density and poor patient prognosis.[129,130] Gene set enrichment studies have demonstrated that ECM in obese patients polarizes macrophages to a stronger M2 phenotype than that of ECM from lean patients.[334] TAMs also express PD-1 that negatively correlates with anti-tumor function.[345]

The administration of αPD-1 *in vivo* improves macrophage anti-tumor function and reduces tumor growth.[345] Given the importance of TAMs in tumor progression, the potential for αPD-1 treatments to restore macrophage anti-tumor function, and the association of a stronger M2 phenotype in obesity, we hypothesize that the macrophages will play a significant role for immunotherapy efficacy in obesity. This chapter focuses on how obesity-induced immune changes, particularly in macrophages, affect tumor growth and immunotherapy efficacy.

Methods

Mice.

Mice were housed in pathogen-free facilities in ventilated cages with 5 animals per cage. All mouse studies and procedures were performed under Institutional Animal Care and Utilization Committee (IACUC)-approved protocols from Vanderbilt University. C57BL/6 mice were obtained from the Jackson laboratory at 3 weeks of age. Diet-induced obese (DIO) and control mice were generated by feeding mice with an open-source purified diet consisting of either 60% fat (D12492 Research Diets, Inc) or continued on standard housing diet (LabDiet Rodent 5001) with 10.7% fat when mice were 5 weeks old, respectively. Mice were maintained on their respective diet for 12 weeks before initiating tumor growth studies or immune analyses.

Tumor cell line and treatment.

The murine colorectal cell line MC38-CEA1 was purchased from Kerafast. C57BL/6 mice were injected subcutaneously in the right flank with 1 x 10^5 MC38-CEA1 cells in 200 μL PBS for the tumor growth studies in DIO and lean mice and 2.5 x 10^5 MC38-CEA1 cells in 200 μL PBS for immunotherapy studies. Tumors were measured with digital calipers every

2-3 days, and tumor volume was calculated as length (mm) × width^2 (mm) × 0.5. In immunotherapy studies, C57BL/6 MC38-CEA tumor bearing mice received either intraperitoneal injections of 200 μg anti-mouse PD-1 antibody (RMP1-14, BioXCell) in 200 μL PBS or 200 μg rat IgG2a isotype control (2A3, BioXCell) in 200 μL PBS on days 5, 7, 9, 11, 13, and 15.

Tumor Dissociation.

To prevent tumors from growing past the 2 cm limit per the IACUC protocol, tumors were collected on day 16 days post-injection. Fresh tumors were first processed with mechanical dissociation, followed by enzymatic digestion with 9.28 mg/mL DNase I (Sigma D5025) and 0.1 g/mL collagenase IA (Sigma C2674), for 1 h at room temperature using a dissociator (Miltenyi) with gentleMACS C-tubes. To remove calcium, cells were resuspended for 5 min in HBSS without calcium or magnesium (Gibco), then resuspended in 5 mM of ethylenediaminetetraacetic acid (EDTA) for 30min at room temperature. Next, cells were passed through a 70 μm filter before ammonium-chloride-potassium (ACK) lysing buffer (KD Medical Inc) was added to remove red blood cells before flow cytometry. Immediate staining was performed for surface marker expression analysis by flow cytometry.

Mouse flow cytometry

One million cells of each tumor or spleen were transferred to a 96-well round-bottom, micro test plate and pelletized at 1500 rpm (524 g) for 5 min (Beckman-Coulture Allegra X-14 Centrifuge). A fixable viability dye (eBioscience, eFluor 780) was used to identify live cells. The following antibodies were used for surface staining: CD45 BV510 (Biolegend, Clone: 30-F11), CD3 FITC (Thermofisher, Clone: 17A2), CD4 PECy5

(Thermofisher, Clone GK1.5), CD8a eFluor 450 (Thermofisher, Clone: 53–6.7), CD279 (PD-1) APC (Thermofisher, Clone: J43), CD279 (PD-1) PE (Biolegend, Clone: RMP1-14), CD44 PECy7 (Thermofisher, Clone: IM7), Foxp3 PE (Thermofisher, Clone: FJK-16S), CD11b eFluor 450 (Thermofisher, Clone: M1/70), F4/80 FITC (Thermofisher, Clone: BM8), CD206 APC (Biolegend, Clone: C068C2), CD86 PE (BD Biosciences, Clone: GL1), iNOS2 PE (Thermofisher, Clone: CXNFT), I-A/I-E (MHCII) (Biolegend, Clone: M5/114.15.2). Briefly, cells were treated with Fc blocking antibodies (TruStain FcX Biolegend) for 10 min at 4 °C followed by cell surface antibodies in FACS Buffer (PBS with 2% FBS) for 30 min at 4 °C. For T cell intracellular staining the FoxP3/Transcription Factor Staining kit (ThermoFisher) was used. The Cytofix/Cytoperm Fixation and Permeabilization Solution Kit (BD Biosciences) was used for macrophage intracellular staining. Cells were pelletized at 1500 rpm (524 g) for 5 min before re-suspending in 200 μL of FACS Buffer. Expression of immune cell surface markers was measured by fluorescence cytometry (MACSQuant, Miltenyi Biotec) and analyzed by FlowJo software (Tree Star Inc.). To select immune cells, live cells were first gated on a fixable viability dye, then a CD45+ cell gate was applied before selecting immune cell subtypes (**Supplemental Figure A.S1**). Some tumor samples did not have enough cells for multiple flow cytometry panels.

Statistics.

Prism software (GraphPad Software Inc.) was used to create graphs and conduct statistical analyses. Data were expressed as mean ± standard error of the mean (s.e.m.). For analysis of three or more groups, a one-way analysis of variance (ANOVA) tests were performed with Tukey post-hoc test, where Gaussian distribution and equal standard deviation as

assumed. For tumor growth, repeated measures two-way analysis of variance (ANOVA) was used with the Geisser-Greenhouse correction as sphericity (equal variability of differences) was not assumed. The two-way ANOVA used the Tukey's multiple comparisons test, with individual variances computed for each comparison. The differences between two test groups was performed using the Mann-Whitney test as Gaussian distributions was not assumed. Differences between more than two test groups was determined using an ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test and a single pooled variance. p values were considered statistically significant if $p < 0.05$.

Ethics Statement

All animal experiments described herein were carried out according to protocols approved by Vanderbilt University's Institutional Animal Care and Use Committee, and all studies followed the National Institutes of Health's guidelines for the care and use of laboratory animals.

Results

Obesity Induces T-Cell Exhaustion

To understand the immune profile differences between DIO and lean mice at baseline, the spleens from non-tumor bearing obese and lean mice were characterized. Five-week old C57BL/6 male mice were fed either a low-fat diet (LFD) or high fat diet (HFD) for 12 weeks over which time the DIO mice gained a significantly larger amount of weight compared to the lean mice (**Figure 2.1A**). Given that obesity causes chronic-low grade inflammation, ongoing inflammation was quantified with markers of T cell activation and exhaustion.[115,117] There was no significant difference, however, between the frequency of $CD8^+$ or $CD4^+$ splenic T cells in DIO and lean mice (**Figure 2.1B, Supplemental Figure A.S2A**). To assess T cell activation, the CD44 surface marker was measured, which is elevated on effector T cells.[346] More T cells expressing the CD44 marker of activation were measured among DIO mouse splenic T cells compared to lean mouse splenic T cells (**Figure 2.1C, Supplemental Figure A.S2B**). Additionally, the chronic inflammatory state and activation of immune cells in obesity may upregulate exhaustion markers, including PD-1. A greater frequency of DIO mouse splenic T cells have elevated levels of PD-1 compared to LFD mouse splenic T cells (**Figure 2.1D, Supplemental Figure A.S2C**). Together, these data support previous findings and suggests that obesity induces an exhausted T cell phenotype.[214] The presence of activation and checkpoint blockade markers on T cells in DIO mice suggests that the obese mice have an elevated inflammatory profile that may influence tumor growth and response to immunotherapy.

Obesity Induces T-Cell Exhaustion

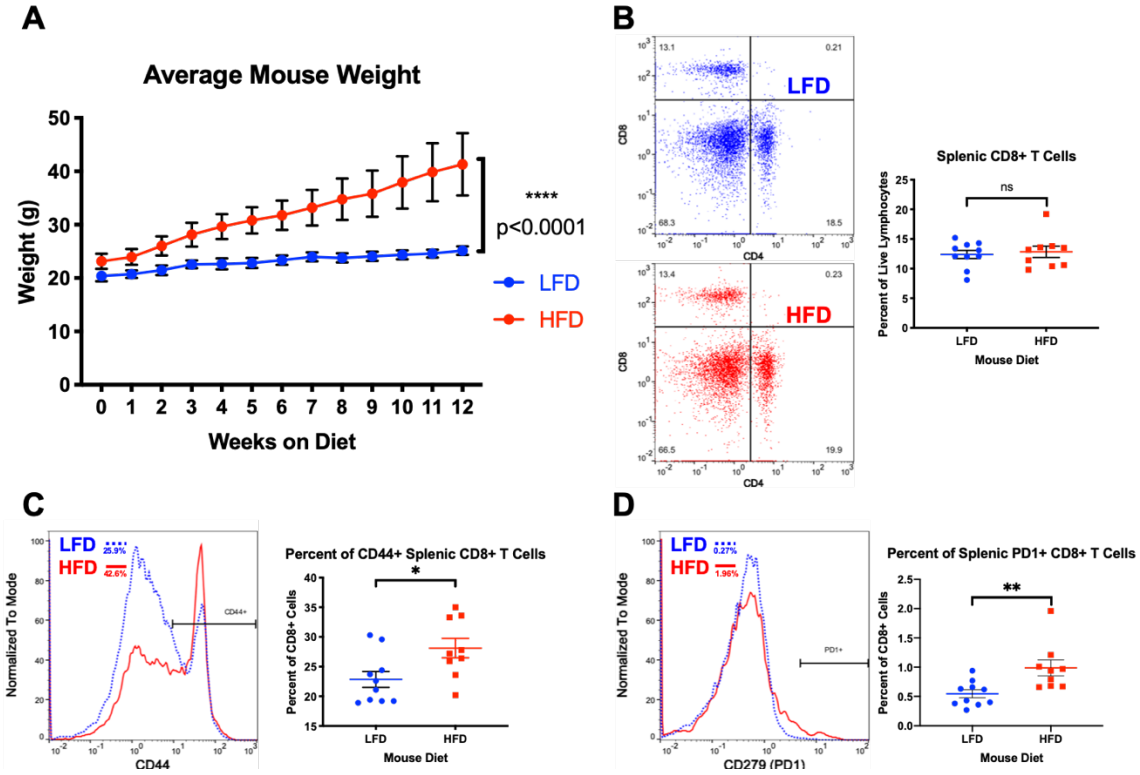

Figure 2.1. (A) Five-week old C57BL/6 male mice were maintained on a control standard chow diet (n=8) or 60kcal high-fat diet (n=8) for 12 weeks and were weighed weekly. Two-way ANOVA with Tukey post-hoc test *p* values used. (B-D) Spleens from mice on their respective diet for 12 weeks were processed into single cell suspensions and were analyzed by flow cytometry for cytotoxic T cells. Representative flow plots and frequency from low-fat diet (LFD) (n=10) and high-fat diet (HFD) (n=9) mouse spleens. (B) Frequency of $CD8^+$ splenic T cells. (C-D) Frequency of $CD44^+$ and $PD\text{-}1^+$ splenic $CD8^+$ T cells. Two-tailed Mann Whitney test *p* values shown. $*p < 0.05$; $***p < 0.001$; $****p < 0.0001$; ns, not significant.

Obesity Increases Tumor Growth and Decreases Anti-Tumor Immune Cells

After determining the differences in immune profiles at baseline, the next studies aimed to identify if the splenic immune cell profiles from DIO and lean mice matched those of tumor-infiltrating immune cells. To accomplish this, the MC38-CEA1 colon cancer model was chosen because it is compatible with the C57BL/6 DIO model and is immunoresponsive to αPD-1 antibody treatment.[347] Despite the increased immune

activation in obesity, MC38-CEA1 subcutaneous tumors grew significantly larger in DIO mice compared to lean mice (**Figures 2.2A, 2.2B**). Different from non-tumor bearing DIO mice, tumor-bearing DIO mice had fewer cytotoxic T cells in their spleens than lean tumor-bearing control mice (**Figure 2.2C**). T cell activation and exhaustion characteristics in tumor-bearing mice were the same as the T cells of non-tumor bearing mice in which the $CD8^+$ T cells had elevated levels of CD44 and PD-1 (**Figure 2.2D, 2.2E**). In addition, tumor-bearing DIO mice had fewer $CD4^+$ T cells compared to lean mice, and there was a greater frequency of PD-1^+ $CD4^+$ T cells in tumor-bearing DIO mice compared to lean mice (**Supplemental Figure A.S3A, A.S3B**). $CD45^+$ immune cells trended to be less frequent in the DIO mouse tumors compared to lean mouse tumors (**Figure 2.2F**). Similar to findings in the splenic T cells from non-tumor bearing mouse, a greater percentage of tumor-infiltrating lymphocytes (TILs) in DIO mice expressed CD44 than in lean mice (**Figure 2.2G**). While DIO mice had an increased frequency of $PD1^+CD4^+$ TILs, PD-1 expression on $CD8^+$ TILs did not significantly differ between obese and lean mice (**Supplemental Figure A.S3C, A.S3D**). Similar changes to the immune system were identified in a DIO model with female C57BL/6 mice bearing E0771 orthotopic breast cancer tumors (**Supplemental Figure A.S4A**). The E0771 tumors were larger in the DIO mice, both by volume and weight (**Supplemental Figure A.S4B, A.S4C**). Splenic $CD8^+$ T cells in the DIO female mice were less frequent in the spleens compared to lean female mice spleens. Additionally, trends suggested an increase in $PD1^+$ T cell frequency in the DIO female mice splenic T cells compared to lean female mice splenic T cells (**Supplemental Figure A.S4D**). Last, the DIO E0771 tumors had a trend of fewer T cells compare to lean E0771 tumors (**Supplemental Figure A.S4E**). Compared to the DIO MC38-CEA1 model in male mice, the female mice did not gain as much weight nor were

the tumors as large, which may cause less significant changes between the obese and lean mouse spleens.

After identifying only modest differences in TILs between obese and lean mice, TAMs were assessed for possible roles in the differences in growth rates between tumors in obese and lean mice. A greater frequency of TAMs from LFD mice expressed inducible nitric oxide synthase 2 (iNOS2) compared to TAMs from HFD mice (**Figure 2.2H**). As a hallmark of M1 macrophages, the trend of increased iNOS expression among LFD TAMs suggests stronger M1 polarization of TAMs from lean mice compared to DIO mice.[348] Unlike the T cells in the spleens, the TILs in the DIO tumor model did not exhibit a stronger exhausted phenotype than lean mice. However, tumors from obese mice trended to possess fewer immune cells and the TAMs from obese mice had a weaker M1 phenotype, suggesting that the TAMs contributed to the significant difference in tumor size between the obese and lean mice.

Obesity Increases Tumor Growth and Decreases Anti-Tumor Immune Cells

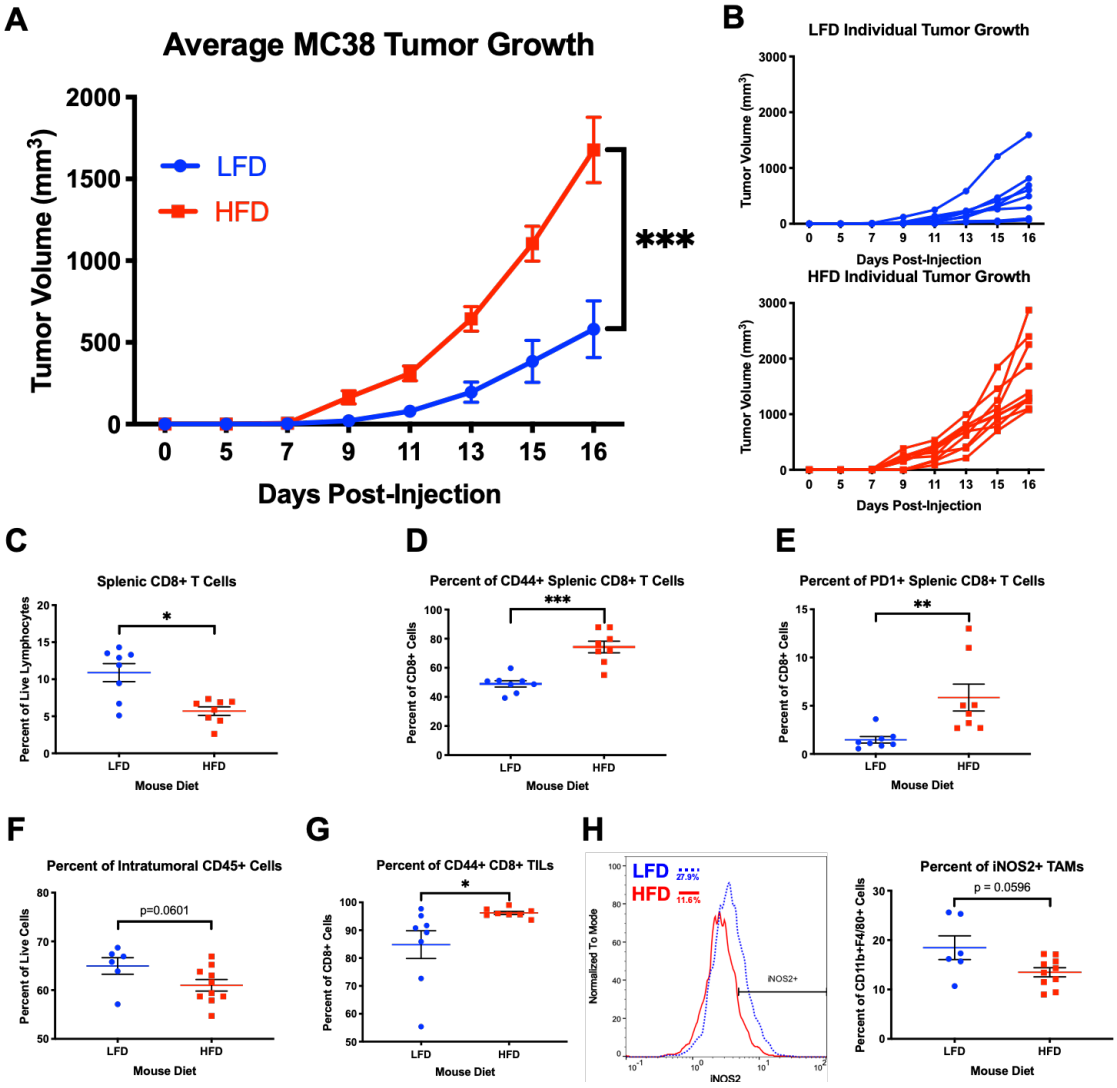

Figure 2.2. (A, B) C57BL/6 male mice on a control standard chow diet (n=10) or 60 kcal high-fat diet (n=10) for 12 weeks were injected with 10^5 MC38-CEA1 cells in the right flank. Two-way ANOVA with Tukey post-hoc test *p* values were used. (C-E) Spleens from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed into single cell suspensions before flow cytometric analysis. Frequency from low-fat diet (LFD) (n=8) and high-fat diet (HFD) (n=8) mouse spleens. (C) Frequency of $CD8^+$ splenic T cells. (D-E) Frequency of $CD44^+$ and $PD1^+$ splenic $CD8^+$ T cells. (F-H) Tumors from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed into single cell suspensions before flow cytometric analysis. (F) Frequency of $CD45^+$ cells from LFD tumors (n=6) and HFD tumors (n=10). (G) Frequency of $CD44^+$ cytotoxic tumor-infiltrating lymphocytes (TILs) from LFD tumors (n=8) and HFD tumors (n=8). (H) Frequency of $iNOS2^+$ tumor-associated macrophages from LFD tumors (n=6) and HFD tumors (n=10). Data are shown as mean± S.E.M., with all individual points shown. Two-tailed Mann Whitney test *p* values shown. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$

Diet-Induced Obese and Lean Mice Both Respond to Immunotherapy, while Only TAMs from DIO Mice Repolarize to "M1" Phenotype

Given the lack of differences in $CD8^+$ TIL but changes in TAMs between obese and lean mouse tumors, both T cells and macrophages were investigated when testing the effects of obesity on immunotherapy efficacy. Lean and obese mice with subcutaneous MC38-CEA1 tumors were treated with αPD-1 antibodies starting on day 5 post-injection and continued every two days until sacrifice on day 16. Obese and lean mice and lean mice treated with αPD-1 antibodies had significantly smaller tumors than the control IgG treated mice, but the change in tumor volume was larger for the obese mice (**Figures 2.3A, 2.3B**). Of note, although the obese mice had a larger decrease in volume than the lean mice, the proportion of volume change with respect to control tumors is not different between obese and lean mice (**Supplemental Figure A.S5A, A.S5B**). The cytotoxic T cells from obese mouse

Diet-Induced Obese and Lean Mice Both Respond to Immunotherapy

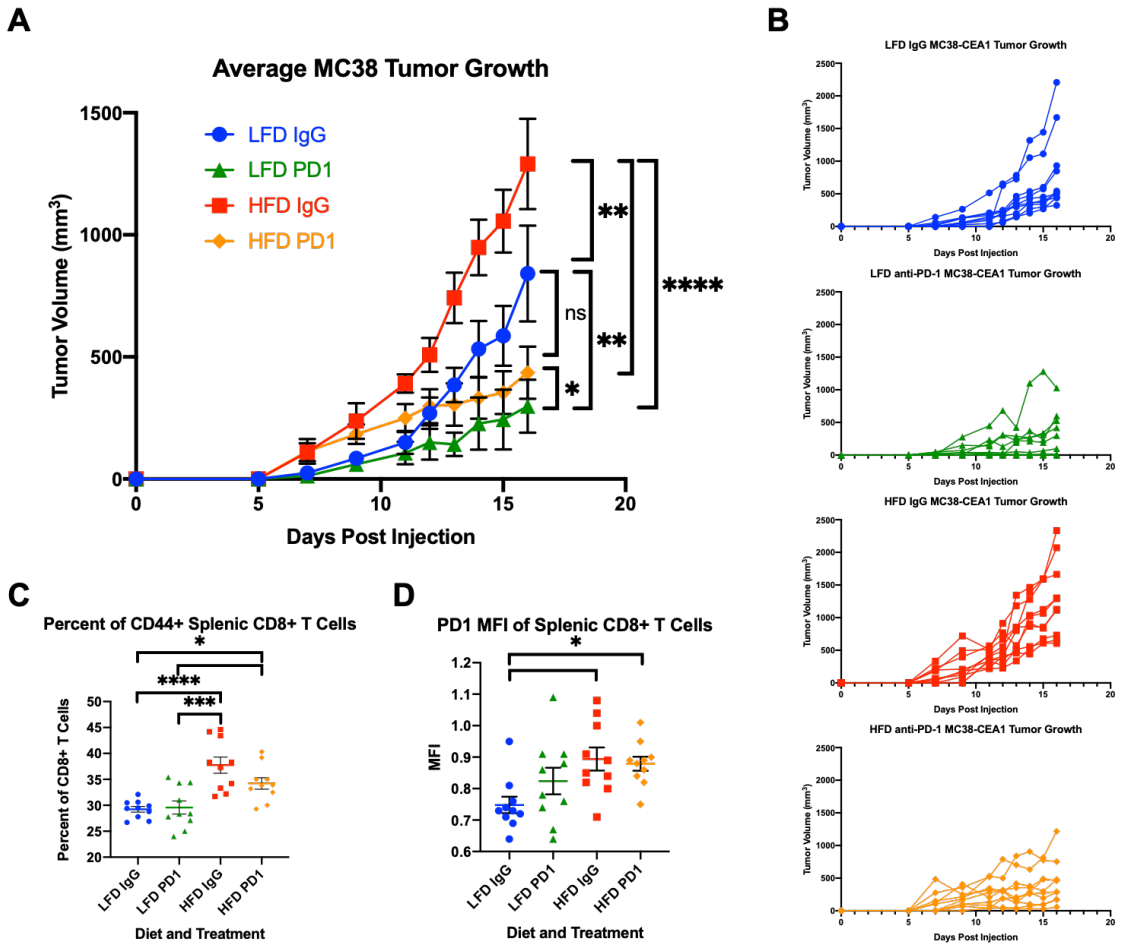

Figure 2.3. (A, B) C57BL/6 male mice on a control standard chow diet (n=10) or 60 kcal high-fat diet (n=10) for 12 weeks were injected with 2.5 x 10^5 MC38-CEA1 cells in the right flank. On day 5 post tumor cell-injection, mice were injected with either 200 µg IgG control antibody or αPD-1 antibody. The injections continued every two days until tumors were collected on day 16 post-injection. Two-way ANOVA with Tukey post-hoc test *p* values were used. (C-D) Spleens from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed into single cell suspensions before flow cytometric analysis. Frequency from LFD IgG (n=10), LFD PD1 (n=10), HFD IgG (n=10), and HFD PD1 (n=10) mouse spleens. (C) Frequency of $CD44^+$ on splenic $CD8^+$ T cells. (D) Expression of PD-1 on splenic $CD8^+$ T cells. Data are shown as mean± S.E.M., with all individual points shown. Ordinary one-way anova test *p* values shown. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant.

spleens had the same increased frequency of $CD44^+$ cells and the same increased PD-1 expression levels (**Figures 2.3C, 2.3D**, **Supplemental Figure A.S6A, A.S6B**) as observed in previous non-tumor and tumor-bearing DIO models (**Figures 2.1C, 2.1D, 2.2D, 2.2E**).

αPD-1 treatment significantly reduced PD-1 levels measured on cytotoxic TILs relative to IgG treated lean and obese mice, consistent with successful blockade of the PD-1/PDL-1 interaction (**Figure 2.4A**). Functional PD-1 blockade failed to modulate CD44 expression frequency on T cells in the spleens of DIO mice in the presence or absence of tumor.

Changes in TAM polarization were among the only significant differences measured in the tumor immune microenvironment; no significant differences in T cell population were detected. Among IgG treated mice, tumors from the obese group had a significantly smaller fraction of TAMs expressing MHCII than tumors from lean mice. Obese mice treated with αPD-1 had a significant increase in the fraction of TAMs expressing MHCII compared to IgG controls. αPD-1 treatment of lean mice, however, did not yield a significant difference in the percent of TAMs expressing MHCII between comparted to matched IgG control-treated mice (**Figure 2.4B**). The TAMs in the DIO mouse model with no treatment had a lower percentage of iNOS2 producing or M1 "anti-tumor" phenotype macrophages compared to lean tumor-bearing mice. However, TAMs from obese mice treated with αPD-1 had a stronger repolarization towards an M1 "anti-tumor" phenotype as indicated by the percent of TAMs expressing MHCII. IFNγ is a cytokine that classically polarizes macrophages to an M1 phenotype, but there was no significant difference in the percent of IFNγ producing $CD8^+$ TILs between diet or treatment groups. These findings suggest that immunotherapy is not only affecting the TILs, but also influencing and repolarizing the TAMs and that this effect may be exacerbated in obesity. The mechanistic details of TAM polarization changes in lean and obese mice treated with αPD-1 remain unknown, including the relative roles of direct or indirect modulation. While there was no significant difference in PD-1 expression on $CD8^+$

TILs, obese and lean mice had different rates of immunotherapy efficacy (**Figure 2.3A, Supplemental Figure A.S2D**). This suggests that immunotherapy efficacy may not be predictable from PD-1 expression on $CD8^+$ TILs alone. These data suggested that the TAMs not only promote tumor growth in obesity but also promote immunotherapy efficacy and subsequent anti-tumor immunity.

Tumor-Associated Macrophages Repolarize in Diet-Induced Obese αPD-1 Treated Mice

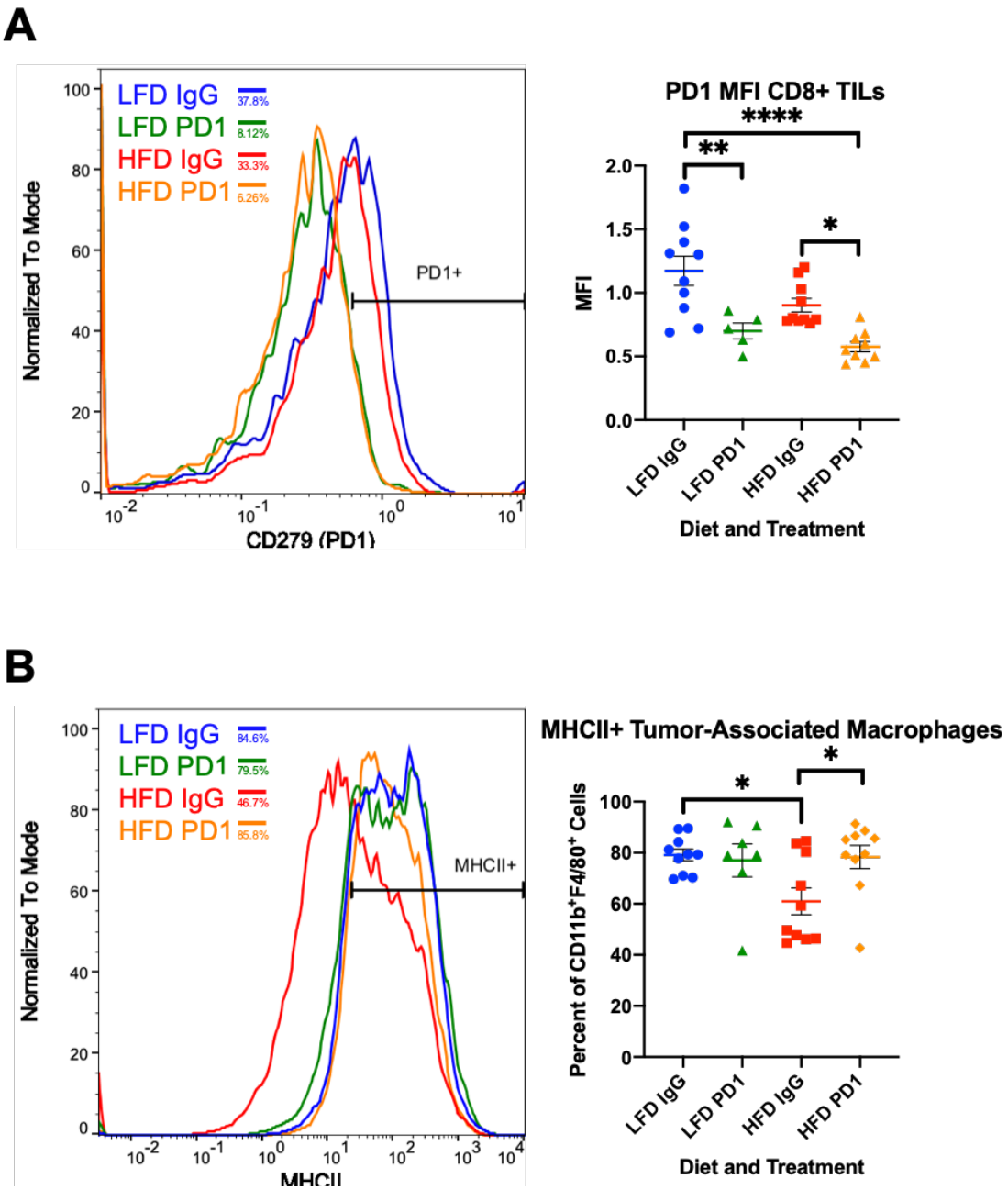

Figure 2.4. (A, B) MC38-CEA1 tumors from LFD IgG (n=10), LFD PD1 (n=10), HFD IgG (n=10), and HFD PD1 (n=10) mice collected 16 days post-injection were processed into single cell suspensions and analyzed by flow cytometry. (A) Expression of PD1 on $CD8^+$ TILs (B) Frequency of $MHCII^+$ on $CD11b^+F4/80^+$ tumor-associated macrophages. Data are shown as mean± S.E.M., with all individual points shown. Ordinary one-way ANOVA test *p* values shown. $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$; ns, not significant.

Discussion

Our findings demonstrate that TAM repolarization enhances immunotherapy efficacy in obesity, which may provide a novel approach to enhancing immunotherapy. In our MC38-CEA1 DIO model, splenic $CD8^+$ T cells had elevated PD-1 frequencies compared to lean mouse splenic T cells, but no differences in PD-1 frequencies of $CD8^+$ TILs were observed. Despite this, the obese mice still experienced a greater decrease in overall tumor volume following immunotherapy compared to lean mice, which are findings similar to those in other preclinical and clinical models.[202,214] Our studies did not identify differences in TILs between obese and lean mice, suggesting that a different immune cell was responsible for the obesity-associated improvement in immunotherapy response. Flow cytometric analyses revealed significant changes in the TAM populations between obese and lean mice. Previous pre-clinical studies recognized that further investigation was needed to identify how TAMs from obese and lean mice modulated immunotherapy efficacy, and recent retrospective clinical studies identified that macrophage polarization improved prediction of immunotherapy efficacy.[199,214]

Our study continues the research effort in understanding the role of macrophages in immunotherapy treatment and initiates studies elucidating additional mechanisms for increased immunotherapy efficacy in obesity. Macrophages are of particular interest in obese patients because their TAMs have a stronger M2 phenotype.[334] The M2-like TAMs increase tumor initiation, progression, and metastasis rates.[140,349] Macrophage polarization occurs on a spectrum where macrophages can exhibit both M1 and M2 functions or surface markers to various degrees. As such, the generalization of macrophages as being distinctly M1 or M2 representing an oversimplification of macrophages' phenotyping.[350–353]

However, given that the functions of M1 macrophages are pro-inflammatory/anti-tumor and M2-macrophages are wound-healing/tumor-promoting, the shorthand descriptions of M1 or M2 macrophage polarization is a useful construct with the understanding that the overall macrophage population is skewed towards an M1 or M2 phenotype.[354] Our studies in obese mice correlated increased tumor progression with decreased M1 TAM phenotype. Tumors with suppressed M1 and/or elevated M2 TAMs are generally considered to have a high potential for progression, regardless of obesity status. Since most tumors possess TAMs that are biased toward M2, TAM ablation has been noted to suppress progression. Our findings of TAM polarization correlating with progression are consistent with previous studies, but extend our TAM knowledge to include a mechanism for increased tumor progression in obese mice.

DIO mice treated with control IgG antibody had a weaker "M1" macrophage phenotype compared to the IgG treated lean mice. However, TAMs from DIO mice treated with αPD-1 had similar "M1" phenotypes as the TAMs from lean mice. Of note, the lean mice treated with αPD-1 did not have a significantly stronger phenotype than that of IgG treated lean mice, demonstrating that the macrophage repolarization was unique to the obese mice. Thus, immunotherapy treatment polarized TAMs from DIO mice toward M1 functions to a greater degree than the same treatment in lean mice. This difference may be a clue to our observation that obese mice treated with immunotherapy experienced a larger decrease in tumor volume compared to identically-treated lean mice. αPD-1 therapy was able to equally 'normalize' TAM polarization in obese and lean models despite the greater degree of dysregulated, M2 polarization in TAMs of obese mice. The efficacy of M1 TAMs in direct tumor cytotoxicity relative to their modulation of TIL cytotoxic activity in

the tumor is understudied, in general, and especially so in the context of obesity and immunotherapy; mechanistic studies with this focus are likely to illuminate important factors that control cancer immunotherapy response.

Although increased IFN-gamma production from $CD8^+$ cytotoxic TILs after αPD-1 blockade is a potential mechanism for the TAM repolarization, there were no significant differences in the frequency of cytotoxic TILs, PD-1 expression on TILs, or IFN-gamma production in TILs among diet or treatment groups. While most immunotherapy research has focused, justifiably, on T cells, new studies recognize the potentially significant role of PD-1/PD-L1 interaction on macrophages and immunotherapy response. Both human and mouse tumors express high levels of PD-1, and PD-1 expression on TAMs inhibits "M1" functions, such as phagocytosis.[345] If obese mice have elevated levels of PD-1 on TAMs as they do in splenic T cells, then TAM phagocytosis in obese mice would be inhibited to a greater extent than that of lean mice. This may be one mechanism for weaker M1 TAM polarization in DIO mice compared to TAMs in lean mice. Regardless of the mechanism, there is a correlation between immunotherapy responders and the presence of M1-like TAMs.[201] Previous studies have shown that TAMs can play a non-immunological role and inhibit αPD-1 therapy by phagocytosing the αPD-1 antibodies, decreasing their efficacy.[355] Further mechanistic studies are needed to identify if enhanced αPD-1 treatment efficacy in obesity is directly achieved through binding upregulated PD-1 on M2-like TAMs or if αPD-1 treatment efficacy is indirectly decreased in lean mice through M1 macrophage phagocytosis of αPD-1 antibodies. Our studies suggest that immunotherapy polarizes TAMs toward M1 behaviors to provide a strong anti-tumor function. TAMs expressing M1 characteristics may reduce tumor growth through at least two mechanisms: 1) reduction of

pro-tumor cytokines and growth factors from M2 macrophages that promote tumor progression and 2) synthesis of anti-tumor cytokines and phagocytosis, resulting in Th1-type cytotoxic T cells and direct cancer cell killing by M1 macrophages.

Conclusion

Altogether, our data provide novel evidence that the polarization of TAMs in obesity significantly increases tumor growth or enhances immunotherapy. While obese mice possessed increased tumor burden and decreased M1 phenotype of TAMs compared to lean mice, immunotherapy treatment in obese mice repolarizes the TAMs to an M1 phenotype, which resulted in a larger decrease in tumor burden than in lean mice. Importantly, lean mice given αPD-1 treatments alone did not experience macrophage repolarization, suggesting a unique component in obesity that is necessary for macrophage repolarization. Further studies are needed to identify how obesity induces TAM repolarization with immunotherapy treatment. By identifying this mechanism, we can target TAM repolarization as a method to enhance immunotherapy efficacy for all patients.

III. THE EFFECTS OF LEPTIN ON TUMOR IMMUNE MICROENVIRONMENT AND POTENTIAL FOR LEPTIN TO ENHANCE IMMUNOTHERAPY EFFICACY

Abstract

Leptin is a pro-inflammatory adipokine that is increased in obesity and may mediate enhanced anti-tumor immunity in obese mice treated with immunotherapy. To test the effect of leptin on tumor growth and anti-tumor immunity, elevated leptin levels were induced in lean mice by providing two weeks of leptin injections before mice received subcutaneous injections of MC38-CEA1 colon cancer cells. Surprisingly, leptin had single-agent preventative and therapeutic anti-tumor efficacy similar to PD-1 checkpoint therapy. Similar to obese mice, the leptin treated mice had stronger M1 polarization of TAMs compared to TAMs from lean mice. We hypothesized that the increase in M1 macrophage polarization would enhance immunotherapy efficacy. Mice treated with both leptin and αPD-1 antibodies had the smallest tumors and greatest M1 macrophage polarization compared to control mouse tumors and leptin or αPD-1 as monotherapies. These data demonstrate that leptin is capable of TAM repolarization to an M1 phenotype that can promote immunotherapy efficacy and may be the mechanism for TAM repolarization in obesity.

Introduction

Our studies in the first aim identified that immunotherapy treatment is more efficacious in obese mice and repolarizes obese mouse TAMs to an M1 phenotype. The goal of the second aim is to identify a mechanism by which macrophage polarization occurs in obese, but not lean, mice. We hypothesize that leptin is the adipokine responsible for repolarizing TAMs to a M1 phenotype, subsequently decreasing tumor size, and potentially enhancing immunotherapy efficacy as was observed in obese mice.

While leptin directs energy homeostasis through the hypothalamus, leptin also activates the immune system and is elevated in obesity as well as in infections and autoimmune diseases.[260,261,270] T cells express leptin receptors that activate STAT3, increasing Th1 phenotype, IFNγ production, and activation markers on $CD4^+$ and $CD8^+$ T cells.[251,356] Leptin decreases the immunosuppressive regulatory T cell population, but this mechanism is not well understood.[223] Through activation of Th1 and $CD8^+$ T cells and inactivation of regulatory T cells, leptin increase pro-inflammatory signals in T cells, contributing to chronic inflammation in obesity.[250]

In macrophages, leptin promotes an M1 phenotype with increased phagocytic function, iNOS expression, and secretion of TNFα, IL-6, and IL-12.[239,241] Studies at the intersection of leptin, obesity, and macrophages have primarily focused on modulation of adipose tissue macrophage inflammation. Leptin deficiency repolarizes pro-inflammatory adipose tissue macrophages to a "M2" phenotype, suggesting that the addition of leptin would promote an M1 phenotype.[357] Here we study how leptin contributes to differences in the tumor immune microenvironment and may enhance immunotherapy efficacy.

Methods

Mice.

Mice were housed in pathogen-free facilities in ventilated cages with 5 animals per cage. All mouse studies and procedures were performed under Institutional Animal Care and Utilization Committee (IACUC)-approved protocols from Vanderbilt University. C57BL/6 mice were obtained from the Jackson laboratory at 3 weeks of age. Diet-induced obese (DIO) and control mice were generated by feeding mice with an open-source purified diet consisting of either 60% fat (D12492 Research Diets, Inc) or continued on standard housing diet (LabDiet Rodent 5001) with 10.7% fat when mice were 5 weeks old, respectively. Mice were maintained on their respective diet for 12 weeks before initiating tumor growth studies.

Leptin measurements.

Plasma leptin concentrations were measured using Leptin Mouse Quantikine ELISA kit as per the manufacturer's instructions (R&D systems).

Tumor cell line, leptin, and immunotherapy treatments.

For leptin studies, recombinant leptin (R&D Systems) was injected at a 1 μg/g body weight concentrations in 200 μL of PBS, twice daily, while control mice received 200 μL of PBS. All leptin experiments were initiated when mice were 5 weeks old. Chronic leptin experiments started leptin treatments two weeks before tumor injection and continued treatments throughout tumor growth. Acute leptin experiments started leptin treatments on day 5 post-tumor injection and continued throughout tumor growth.

The murine colorectal cell line MC38-CEA1 was purchased from Kerafast. C57BL/6 mice were injected subcutaneously in the right flank with 1 x 10^5 MC38-CEA1 cells in 200 μL PBS for chronic leptin treatment studies and 2.5 x 10^5 MC38-CEA1 cells in 200 μL PBS for leptin and immunotherapy studies. Tumors were measured with digital calipers every 2-3 days, and tumor volume was calculated as length (mm) × width^2 (mm) × 0.5. In immunotherapy studies, C57BL/6 MC38-CEA tumor bearing mice received either intraperitoneal injections of 200 μg anti-mouse PD-1 antibody (RMP1-14, BioXCell) in 200 μL PBS or 200 μg rat IgG2a isotype control (2A3, BioXCell) in 200 μL PBS on days 5, 7, 9, 11, 13, and 15.

Tumor Dissociation.

To prevent tumors from growing past the 2 cm limit per the IACUC protocol, tumors were collected on day 16 days post-injection. Fresh tumors were first processed with mechanical dissociation, followed by enzymatic digestion with 9.28 mg/mL DNase I (Sigma D5025) and 0.1 g/mL collagenase IA (Sigma C2674), for 1 h at room temperature using a dissociator (Miltenyi) with gentleMACS C-tubes. To remove calcium, cells were resuspended for 5 min in HBSS without calcium or magnesium (Gibco), then resuspended in 5 mM of ethylenediaminetetraacetic acid (EDTA) for 30min at room temperature. Next, cells were passed through a 70 μm filter before ammonium-chloride-potassium (ACK) lysing buffer (KD Medical Inc) was added to remove red blood cells before flow cytometry. Immediate staining was performed for surface marker expression analysis by flow cytometry.

Mouse flow cytometry.

One million cells of each tumor or spleen were transferred to a 96-well round-bottom, micro test plate and pelletized at 1500 rpm (524 g) for 5 min (Beckman-Coulture Allegra X-14 Centrifuge). A fixable viability dye (eBioscience, eFluor 780) was used to identify live cells. The following antibodies were used for surface staining: CD45 BV510 (Biolegend, Clone: 30-F11), CD3 FITC (Thermofisher, Clone: 17A2), CD4 PECy5 (Thermofisher, Clone GK1.5), CD8a eFluor 450 (Thermofisher, Clone: 53–6.7), CD279 (PD-1) APC (Thermofisher, Clone: J43), CD279 (PD-1) PE (Biolegend, Clone: RMP1-14), CD44 PECy7 (Thermofisher, Clone: IM7), Foxp3 PE (Thermofisher, Clone: FJK-16S), CD11b eFluor 450 (Thermofisher, Clone: M1/70), F4/80 FITC (Thermofisher, Clone: BM8), CD206 APC (Biolegend, Clone: C068C2), CD86 PE (BD Biosciences, Clone: GL1), iNOS2 PE (Thermofisher, Clone: CXNFT), I-A/I-E (MHCII) (Biolegend, Clone: M5/114.15.2). Briefly, cells were treated with Fc blocking antibodies (TruStain FcX Biolegend) for 10 min at 4 °C followed by cell surface antibodies in FACS Buffer (PBS with 2% FBS) for 30 min at 4 °C. For T cell intracellular staining the FoxP3/Transcription Factor Staining kit (ThermoFisher) was used. The Cytofix/Cytoperm Fixation and Permeabilization Solution Kit (BD Biosciences) was used for macrophage intracellular staining. Cells were pelletized at 1500 rpm (524 g) for 5 min before re-suspending in 200 μL of FACS Buffer. Expression of immune cell surface markers was measured by fluorescence cytometry (MACSQuant, Miltenyi Biotec) and analyzed by FlowJo software (Tree Star Inc.). To select immune cells, live cells were first gated on a fixable viability dye, then a CD45+ cell gate was applied before selecting immune cell subtypes. Some tumor samples did not have enough cells for multiple flow cytometry panels.

Statistics.

Prism software (GraphPad Software Inc.) was used to create graphs and conduct statistical analyses. Data were expressed as mean ± standard error of the mean (s.e.m.). For analysis of three or more groups, a one-way analysis of variance (ANOVA) tests were performed with Tukey post-hoc test. For tumor growth, repeated measures two-way analysis of variance (ANOVA) was used with the Geisser-Greenhouse correction as sphericity (equal variability of differences) was not assumed. The two-way ANOVA used the Tukey's multiple comparisons test, with individual variances computed for each comparison. The differences between two test groups was performed using the Mann-Whitney test as Gaussian distributions was not assumed. Differences between more than two test groups was determined using an ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test and a single pooled variance. p values were considered statistically significant if $p < 0.05$.

Ethics Statement

All animal experiments described herein were carried out according to protocols approved by Vanderbilt University's Institutional Animal Care and Use Committee, and all studies followed the National Institutes of Health's guidelines for the care and use of laboratory animals.

Results

Chronic Leptin Treatments Decrease Tumor Growth, Induce DIO Splenic T Cell Activation and Exhaustion, and Promote "M1" TAM Repolarization

Obesity-associated hormones influence immune cell activation and differentiation.[119,327–330] Leptin is an adipokine that has been previously shown to polarize macrophages to an M1 phenotype is correlated with elevated PD-1 levels on $CD8^+$ T cells. Similarly, leptin was significantly elevated in our diet-induced obese mouse model (**Figure 3.1A**).[214,221,239,246,357,358] However, the effects of leptin polarization on TAMs has not been investigated. By using exogenous leptin injections in young, lean mice, the ability of leptin to induce M1-macrophage polarization was studied without additional immune cell changes seen in diet-induced obese models. To simulate the elevated leptins levels in obesity, young, lean mice received leptin or PBS control intraperitoneal injections twice a day for two weeks before injecting the mice subcutaneously with MC38-CEA1 colon cancer cells. Consistent with Leptin as a satiety signal, mice lost weight during the first week of treatment although much of this weight was regained during the second week of treatment (**Figure 3.1B**).[359] Leptin injections continued throughout the tumor growth period, and the mice that received the leptin had significantly smaller tumors than PBS-control treated mice by volume and weight (**Figure 3.1C, 3.1D, Supplemental Figure B.S1A**). The spleens from mice treated with leptin had fewer T cells compared to control mouse spleens (**Figure 3.1E, Supplemental Figure B.S1B**). A greater percent of splenic T cells from leptin-treated mice expressed PD-1 (**Figure 3.1F, Supplemental Figure B.S1C**). The splenic $CD4^+$ T cells from leptin-treated mice had a higher frequency of $CD44^+$ cells (**Supplemental Figure B.S1D**). This splenic T cell profile of lean mice treated with leptin matches that of the diet-induced obese mouse (**Figure 2.1C, 2.1E, 5.1E,**

5.1F, Supplementary Figure B.S1). The myeloid-derived suppressor cells (MDSCs) were also altered by the leptin treatments. The leptin-treated mice had an increased frequency of polymorphonuclear MDSCs (PMN-MDSCs) and a decreased frequency of monocytic MDSCs (M-MDSCs) (**Supplementary Figure B.S1E, B.S1F**). The PMN-MDSCs are a neutrophil-like cell and the M-MDSCs are similar to M2 macrophages, and leptin-induced changes further support an increased pro-inflammatory environment.

The tumor immune cell phenotypes did not match those of the spleen. The TILs from leptin-treated mice did not have elevated PD-1 expression. However, the similarities between leptin-treated and the diet-induced obese mouse did not extend to assessments of monocytes and macrophages. Leptin treatment induced a higher percent of splenic macrophage expression of MHCII and CD86 compared to PBS-control treated mice (**Figures 3.1G, 3.1H**). Additionally, a significantly higher level of MHCII was expressed by TAMs in leptin-treated mice (**Figure 3.1I**), and the TAMs had a trend for higher CD86 expression (**Figure 3.1J**). There was a trend of decreased M-MDSC frequency in the tumors of leptin-treated mice compared to PBS-treated control mice (**Supplementary Figure B.S1G**). No differences were observed in the numbers of TILs between leptin-treated and control mouse tumors. However, leptin repolarized the TAMs to a stronger M1 phenotype.

Chronic Leptin Treatments Decrease Tumor Burden

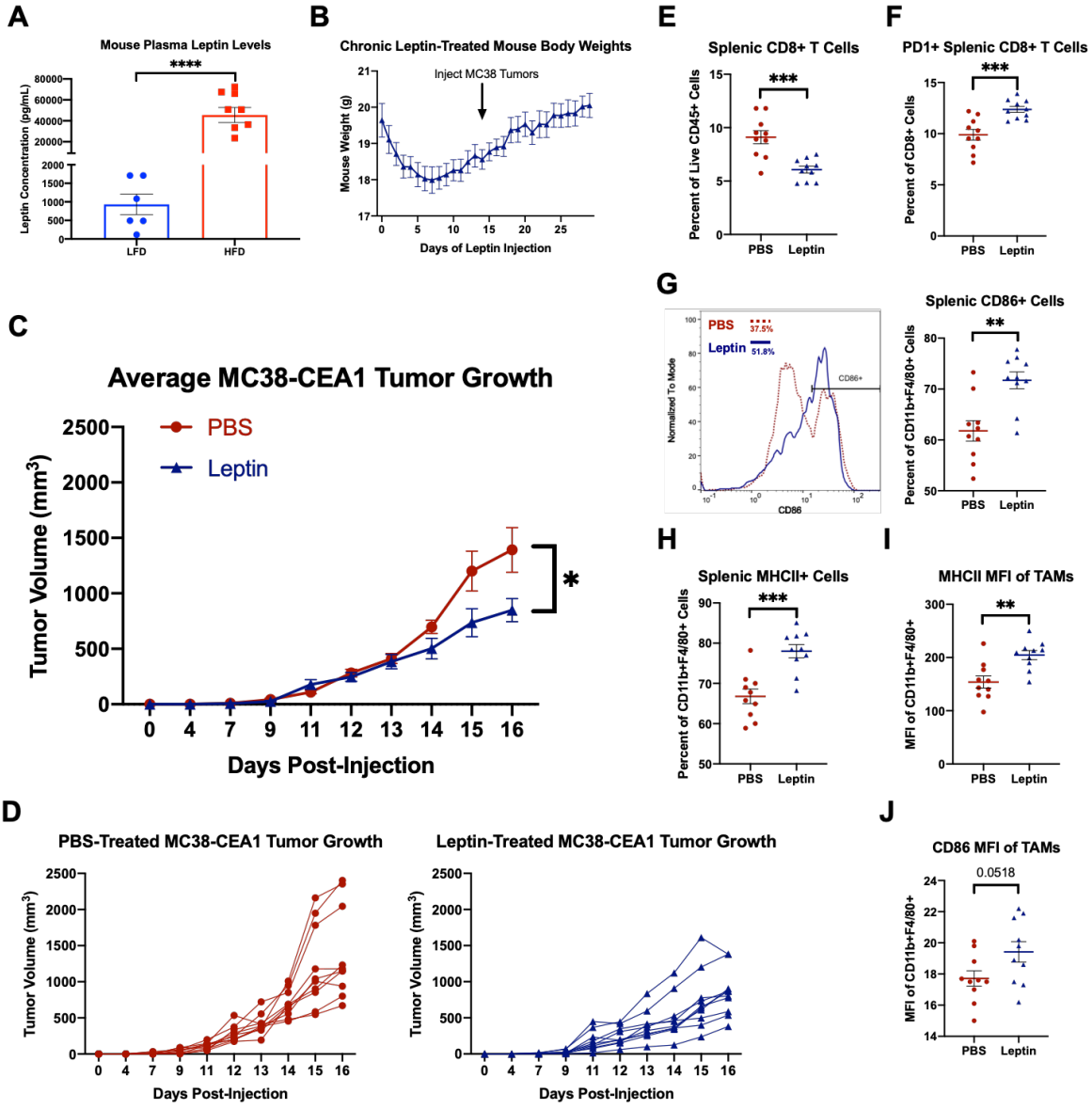

Figure 3.1. (A) Plasma collected from MC38-CEA1 tumor-bearing C57BL/6 male mice on a control standard chow diet (n=6) or 60 kcal high-fat diet (n=8) was measured for leptin by ELISA. Two-tailed Mann Whitney test *p* values shown. (B-D) Five-week old C57BL/6 male mice on a control standard chow diet were injected with either 200 µL of leptin (1 µg/g body weight) or PBS control twice a day for two weeks before subcutaneous injections with 10^5 MC38-CEA1 cells were given in the right flank. Leptin injections continued throughout the tumor growth period. (B) Mice were weighed daily and weights were recorded. (C, D) MC38-CEA1 tumor growth was recorded by digital caliper measurements. Two-way ANOVA with Tukey post-hoc test *p* values used. (E-H) Spleens from mice with MC38-CEA1 tumors were collected 16 days post-injection, processed into single cell suspensions and analyzed by flow cytometry. Frequency from PBS-treated (n=10) and leptin-treated (n=10) mouse spleens. (E) Frequency of $CD8^+$ splenic T cells.

(F) Frequency of $PD1^+$ splenic $CD8^+$ T cells. (G) Frequency of $CD86^+$ cells from $CD11b^+$ and $F4/80^+$ cell population. (H) Frequency of $MHCII^+$ cells from $CD11b^+$ and $F4/80^+$ cell population. Tumors from mice with MC38-CEA1 tumors were collected 16 days post-injection, processed into single cell suspensions, and analyzed by flow cytometry. (I) MHCII expression of tumor-associated macrophages. (J) CD86 expression of tumor-associated macrophages. Data are shown as mean± S.E.M., with all individual points shown. Two-tailed Mann Whitney test *p* values shown. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Acute Leptin Treatments Improve Immunotherapy Efficacy and Repolarizes TAMs to "M1" Phenotype seen in DIO Mice

With the goal of testing if leptin can act as an immunotherapy and improve immunity against established tumors, leptin treatments were initiated simultaneously with PD-1 blockade immunotherapy treatment on day 5 post injection. The same MC38-CEA1 tumor model, immunotherapy, and leptin treatment dosing was used as in other studies here. The leptin IgG, PBS αPD-1, and leptin αPD-1 treatment groups all had significantly smaller tumors than the control PBS IgG treated group (**Figure 3.2A, 3.2B**). The tumor growth curves exhibit a trend for smallest tumor volume in the leptin and αPD-1 co-treatment mice (**Figure 3.2B**). In the spleen, leptin and αPD-1 co-treatment generated the greatest percent of CD86 expressing macrophages and T regulatory cells amongst the non-control groups (**Figure 3.2C, Supplementary Figure B.S2A**).

Acute Leptin and Immunotherapy Treatments Decrease Tumor Burden

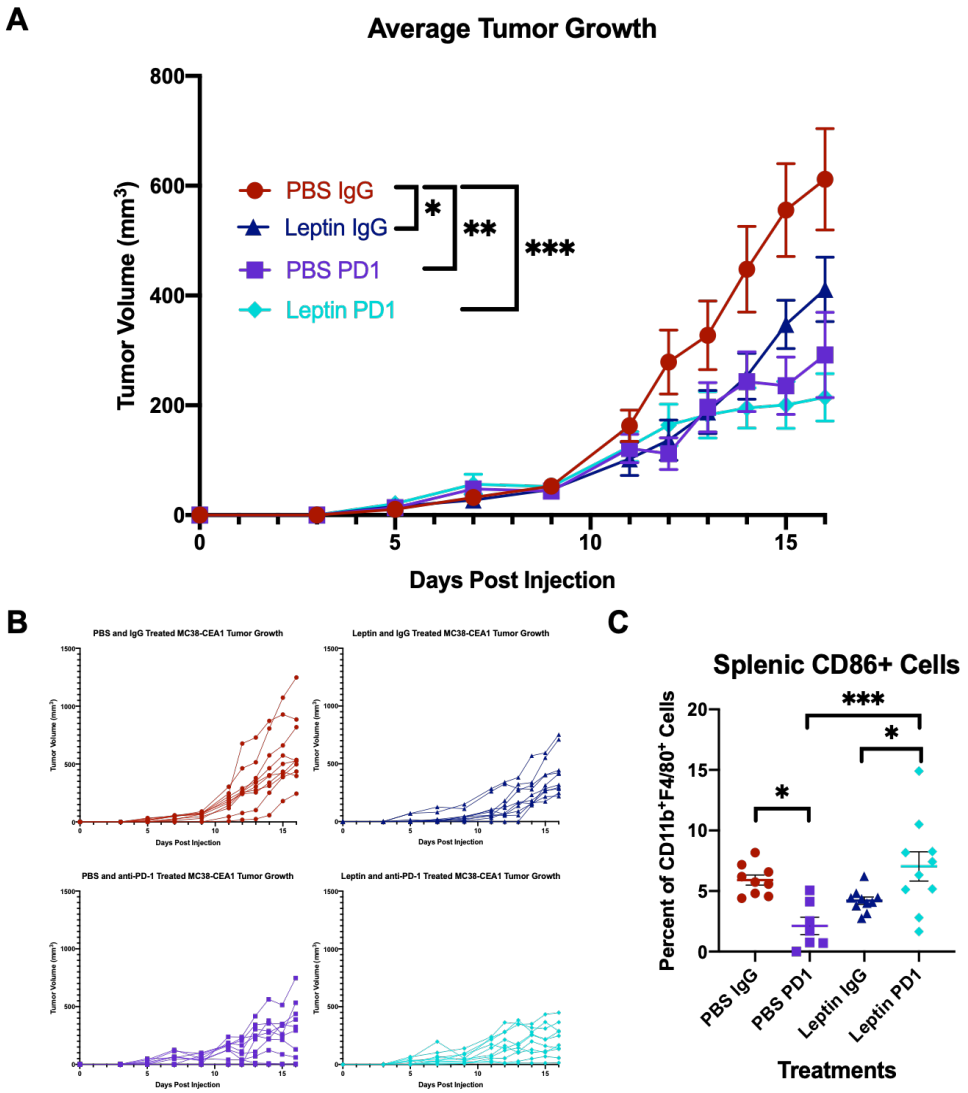

Figure 3.2. (A, B) Five-week old C57BL/6 male mice were given subcutaneous injections with 2.5 x 10^5 MC38-CEA1 cells in the right flank. On day 5 post tumor-injection, mice were injected with either 200 µg IgG control antibody or αPD-1 antibody, and the injections continued every two days. Additionally, on day 5 post-tumor injection, mice received either leptin (1 µg/g body weight) or PBS control twice a day. Treatments for the PBS IgG antibody (n=10), Leptin IgG antibody (n=10), PBS αPD-1 antibody (n=10), and Leptin αPD-1 antibody continued until 16 days post-injection. Two-way ANOVA with Tukey post-hoc test *p* values used. (C) PBS IgG antibody treated (n=9), PBS αPD-1 antibody treated (n=7), Leptin IgG antibody treated (n=10), and Leptin αPD-1 antibody treated (n=10) spleens from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry. Frequency of $CD86^+$ cells from $CD11b^+$ and $F4/80^+$ cell population. Two-tailed Mann Whitney test *p* values shown. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

In the tumor, successful knockdown of PD1 occurred in the αPD-1 and αPD-1 with

leptin treatment groups (**Supplementary Figure B.S2B**). The αPD-1 monotherapy and leptin with αPD-1 co-treatment was the significantly decreased the percent of TAMs (**Figure 3.3A**). The remaining TAMs in the leptin and αPD-1 co-treatment group had the highest "M1 anti-tumor" markers, including both macrophage expression of both MHCII and iNOS2 (**Figure 3.3B, 3.3C**). The αPD-1 treatment group had a significantly greater frequency of $CD8^+$ TILs compared to the control and leptin monotherapy treatment groups, and there was a trend for an increased frequency of $CD8^+$ TILs in the co-treatment αPD-1 and leptin treatment group as well (**Supplementary Figure B.S2C**). Altogether, these findings demonstrate that the repolarization of TAMs to an "M1 anti-tumor" phenotype is correlated with a decrease in tumor size with leptin and αPD-1 treatments. Further, leptin may act as a novel immunotherapy agent to mimic the anti-tumor effects of obesity-related inflammation.

Leptin and Immunotherapy Co-Treatment Repolarizes Tumor-Associated Macrophages to "M1" Phenotype

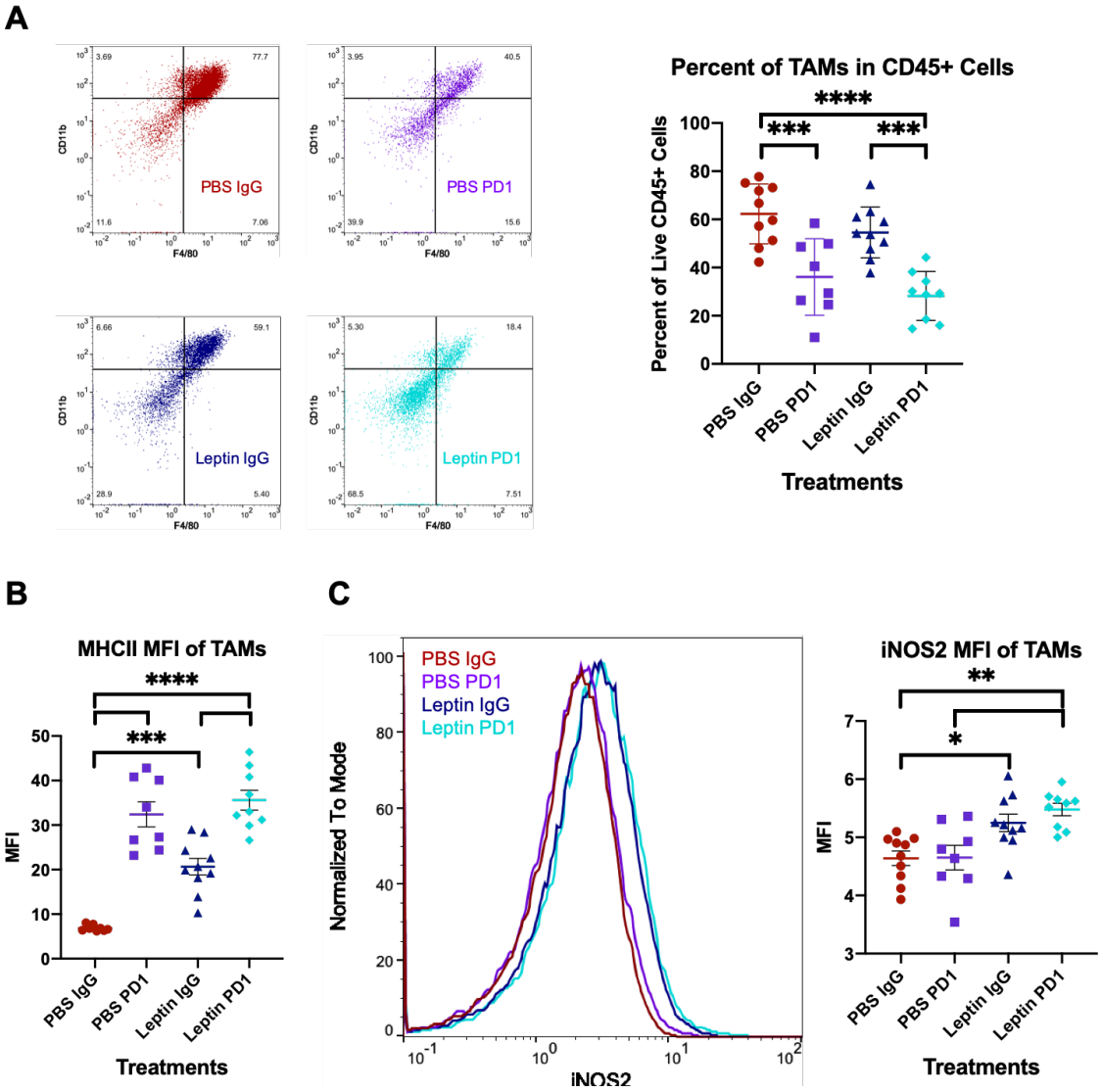

Figure 3.3. (A-C) PBS IgG antibody treated (n=10), PBS αPD-1 antibody treated (n=8), Leptin IgG antibody treated (n=10), and Leptin αPD-1 antibody treated (n=9) tumors from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry. (A) Frequency of $CD11b^+$ and $F4/80^+$ tumor-associated macrophages (TAMs). (B) Expression of MHCII in TAMs. (C) Expression of iNOS2 in TAMs. Two-tailed Mann Whitney test *p* values shown. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, $****p < 0.0001$.

Discussion

Our study aimed to identify if leptin can repolarize TAMs to a M1 phenotype, subsequently decreasing tumor size, and potentially enhancing immunotherapy efficacy as was observed in obese mice. Both T cells and macrophages express leptin receptors that activate STAT3 and STAT1 respectively, and eventually result in PD-1 upregulation, which occurred in splenic T cells and was not tested in macrophages.[356] Others have studied targeted effects of leptin on TILs and the associated decrease in tumor burden, but we believe that this is the first study investigating leptin-induced macrophage changes in an immunotherapy setting.[360]

By providing chronic-leptin treatments, an elevated serum leptin level was achieved in the mice before initiating tumor growth experiments. Chronic leptin-treated mice had significantly smaller tumors, elevated PD-1 and CD44 expression on T cells, and an increased M1-macrophage phenotype. When combining leptin treatments with αPD-1 antibodies, the co-treated mice had the smallest tumors, the greatest decrease in TAM frequency, and the largest increase in M1 polarization compared to either monotherapy and control treated mouse tumors.

From our findings, the DIO immunotherapy treated mice (Chapter II) and the lean leptin and immunotherapy co-treated mice had a very similar immune profiles: decreased tumor burden, elevated PD-1 and CD44 expression on splenic T cells, and repolarized macrophages to an M1 phenotype. Of note, elevated leptin in obesity results in leptin resistance, and if exogenous leptin is provided to DIO mice, the macrophages do not polarize to an M1 phenotype.[279] Therefore, the αPD-1 treatments may allow for leptin

sensitization in immune cells and subsequent TAM repolarization, but this mechanism is not completely described and is a clear focus for further study.

Conclusion

Altogether, our data provide convincing evidence that leptin can modify TAMs to influence tumor progression and immunotherapy. Leptin is capable of both increasing PD-1 expression on T cells and polarizing TAMs towards an M1 phenotype. Consequently, leptin may be responsible for the increased M1 macrophage polarization during αPD-1 antibody treatment of obese mice, as was observed in the experiments with exogenous leptin delivery. Further studies are needed to identify the exact interactions between leptin, TAMs, and immunotherapy. Nevertheless, our studies highlight the importance of leptin and TAMs in immunotherapy efficacy and suggest that leptin-mediated repolarizing of TAMs can increase immunotherapy efficacy.

IV. IMMUNOTHERAPY AND RADIOTHERAPY CAUSES AN ABSCOPAL TREATMENT RESPONSE IN A MOUSE MODEL OF CASTRATION RESISTANT PROSTATE CANCER

Text for Chapter IV taken from:

> **Dudzinski SO,** Cameron BD, Wang J, Kirschner AN. Combination Immunotherapy and Radiotherapy Causes an Abscopal Treatment Response in a Mouse Model of Castration Resistant Prostate Cancer. *Journal for ImmunoTherapy of Cancer 2019*.

Abstract

Prostate cancer is poorly responsive to immune checkpoint inhibition, yet a combination with radiotherapy may enhance the immune response. In this study, we combined radiotherapy with immune checkpoint inhibition (iRT) in a castration-resistant prostate cancer (CRPC) preclinical model. Two Myc-CaP tumor grafts were established in each castrated FVB mouse. Anti-PD-1 or anti-PD-L1 antibodies were given and one graft was irradiated 20 Gy in 2 fractions. In CRPC, a significant increase in survival was found for radiation treatment combined with either anti-PD-1 or anti-PD-L1 compared to monotherapy. The median survival for anti-PD-L1 alone was 13 days compared to 30 days for iRT ($p=0.0003$), and for anti-PD-1 alone was 21 days compared to 36 days for iRT ($p=0.0009$). Additional treatment with anti-CD8 antibody blocked the survival effect. An abscopal treatment effect was observed for iRT in which the unirradiated graft responded similarly to the irradiated graft in the same mouse. At 21 days, the mean graft volume for anti-PD-1 alone was 2094 mm^3 compared to iRT irradiated grafts 726 mm^3 ($p=0.04$) and unirradiated grafts 343 mm^3 ($p=0.0066$). At 17 days, the mean graft volume for anti-PD-L1 alone was 1754 mm^3 compared to iRT irradiated grafts 284 mm^3 ($p=0.04$) and unirradiated grafts 556 mm^3 ($p=0.21$). Flow cytometry and immunohistochemistry identified CD8+

immune cell populations altered by combination treatment in grafts harvested at the peak effect of immunotherapy, 2-3 weeks after starting treatment. These data provide preclinical evidence for the use of iRT targeting PD-1 and PD-L1 in the treatment of CRPC. Immune checkpoint inhibition combined with radiotherapy treats CPRC with significant increases in median survival compared to drug alone: 70% longer for anti-PD-1 and 130% for anti-PD-L1, and with an abscopal treatment effect.

Introduction

Prostate cancer is the most common non-cutaneous malignancy and the second-leading cause of cancer-related mortality among men in the USA, with distant disease having a 5-year survival rate of 29.8%.[361] Though metastatic disease is initially responsive to androgen blockade, over time this treatment selects for a castration-resistant prostate cancer (CRPC) population with modern systemic treatments providing a median survival of 2.8 years, albeit with significant quality of life detriment due to treatment-related effects and disease progression.[362]

The combination of immunotherapy and radiotherapy is an emerging clinical treatment paradigm, a growing research sector, and a critical research domain supported by the Radiation Biology Task Force.[363] X-ray radiation treatment (XRT) can activate both the adaptive and innate immune systems through directly killing tumor cells, causing mutations in tumor-derived peptides, and causing localized inflammation that increases immune cell trafficking to tumors.[307,364] Most importantly, the activated immune system may cause tumor-directed treatment responses away from the site of irradiation, i.e., an abscopal treatment effect, which has the potential to treat disease throughout the body.

However, prostate tumors are considered poorly responsive to immunotherapy due to

their low genetic mutational load, their lack of activated tumor-infiltrating lymphocytes, and specific genetic alterations that influence the immune landscape.[365,366] Studies have shown over 50% of aggressive prostate cancers express high levels of PD-L1, a key factor in suppressing the local immune response.[367] A negative regulator of the immune response, Tregs have also been found to be enriched in both the tumor and peripheral blood of patients with prostate cancer.[368,369] Altogether, prostate cancer has mechanisms to evade and inhibit anti-tumor immunity.

Clinical trials have studied immune checkpoint inhibition for CRPC. One phase III randomized trial of 799 patients tested 8 Gy XRT to a CRPC bone metastasis followed by either placebo or 4 cycles of ipilimumab (anti-CTLA4) and found the combination provided a statistically significant 7 months median survival increase in a post-hoc analysis of a predefined subgroup of patients with low tumor burden (22.7 months vs 15.8 months, p=0.0038).[370] However, only a trend for improved overall survival was seen for the whole cohort (11.2 months vs 10.0 months, p=0.053) and therefore the trial did not provide enough evidence to meet its primary endpoint and influence clinical practice.[370] A subsequent phase III randomized clinical trial for 600 patients with low tumor burden tested ipilimumab versus placebo, without XRT.[371] However, this ipilimumab-only approach failed to show any overall survival benefit and only a marginal progression-free survival benefit and prostate-specific antigen (PSA) response was seen, suggesting that the combination with XRT produces a superior treatment response in patients with low disease burden.[371] These large randomized clinical trials indicate there is a potentially powerful treatment approach when combining radiotherapy with immunotherapy for CPRC, but the optimal treatment combination has not yet been found for most patients to derive benefit.

This project builds upon the findings of these clinical trials to develop preclinical

models that can be used to optimize the treatment approach. Anti-PD-1 and anti-PD-L1 antibodies are immune checkpoint inhibitors that target tumor-immune cell interactions and clinically have a reasonably favorable side-effect profile in patients. This suggests PD-inhibitors may be superior to anti-CLTA4 agents, which primarily block the interaction between immune cells without directly involving the tumor. However, PD-1 agents alone show little response in treating CRPC in early phase clinical trials.[372] Nevertheless, logically following the clinical trials described above, we hypothesized that combination PD-based immunotherapy-radiotherapy (iRT) approach would trigger a robust treatment response against CRPC that is mediated through the immune system, causing both local and distant (abscopal) effects, while likely being better tolerated in patients than an anti-CTLA4 approach.

There is evidence to suggest that the tumor-dependence on PD-1/PD-L1 immunosuppression is enhanced in lesions that respond to radiation.[373] Therefore, we examine a combination of immune checkpoint inhibition and radiotherapy for CRPC that causes local and abscopal treatment effects mediated by activated immune cells.

Materials and Methods

Cell Lines.

Myc-CaP cells were purchased from ATCC, authenticated by short tandem repeat analysis and confirmed *Mycoplasma*-free (CellCheck Mouse Plus, IDEXX BioAnalytics, Columbia, MO), and grown in cell culture in DMEM medium (Corning) supplemented with 10% fetal bovine serum (Corning) and 1% penicillin-streptomycin (Gibco).

Mouse Model of Immune-Radiotherapy.

A mouse prostate cancer model that mimics common human CRPC was developed. Myc-CaP tumors were engrafted into FVB mice (JAX) from which the tumor cells were derived.[374] Injecting one million cells in 50-70% Matrigel (Corning), two subcutaneous tumors were simultaneously established in each mouse, one in the flank and one in the hindlimb (leg). After the tumor grafts reached 500 mm^3, castration was performed, and after brief regression the tumors continued to grow castration-resistant.[375] Mice were then treated with either anti-mouse PD-1 (clone RMP1-14, Bio X Cell) or PD-L1 (clone B7-H1, Bio X Cell) antibody, 0.2 mg IP given on days 0, 2, 4, and 7. Only leg tumors were treated with XRT 20 Gy in 2 equal treatments given on days 7 and 8. Survival was assessed as the primary outcome. A separate cohort was treated similarly and tumors were harvested on days 14-17 for flow cytometry and immunohistochemistry analyses. Treatment cohorts were repeated at least 3 times with adequately powered numbers of mice per group with similar results. Representative data from example cohorts are presented in the figures.

Immunohistochemistry.

Harvested tumor grafts were fixed in 10% zinc-formalin (Fisher Scientific) at room temperature overnight, then transferred to 70% ethanol for paraffin embedding. Immunohistochemical staining for Ki67 and cleaved caspase-3 was performed on serial sections. Whole slide digital imaging was analyzed using QuPath software for positive cell counts, using sigma level 2.0 and threshold level 0.3.[376]

Tumor Dissociation.

To analyze the tumor immune microenvironment during the anticipated efficacious period of immune checkpoint activity, tumors were collected at day 14-17 after starting

immune checkpoint inhibitor. Fresh tumors were dissociated into single cell suspensions with DNAse I (Invitrogen), collagenase Type IV (Sigma), and hyaluronidase (MP Biomedicals) for 1 hour at room temperature using a dissociator (Miltenyi) with gentleMACS C-tubes. To remove calcium, cells were resuspended for 5 minutes in HBSS without calcium or magnesium (Gibco), then resuspended in 5 mM of EDTA for 30 minutes at room temperature. Next, cells were passed through a 70 μm filter before ACK lysing buffer (KD Medical Inc) was added to remove red blood cells before flow cytometry. Immediate staining was performed for surface marker expression to analyze with flow cytometry.

Fluorescence Cytometry.

One million cells of each tumor were transferred to a 96-well round-bottom, micro test plate and pelletized at 1500 rpm for 5 minutes (Beckman-Coulture Allegra X-14 Centrifuge). A fixable viability dye (eBioscience, eFluor 780) was used to identify live cells. The following antibodies were used for surface staining: CD3 APC (Biolegend, Clone: 17A2), CD4 BV510 (BD Bioscience Clone RM4-5), CD8a eFluor 450 (eBioscience, Clone: 53-6.7), CD279 (PD-1) FITC (eBioscience, Clone: J43), CD44 PECy5 (eBioscience, Clone: IM7), CD335 PECy7 (Biolegend, Clone: 29A1.4), CD11b AF488 (Biolegend M1/70), F4/80 BV421 (Biolegend BM8), CD206 PE (Biolegend C068C2), CD86 APC (Biolegend GL-1). Briefly, cells were stained with Fc blocking antibodies (TruStain FcX Biolegend) for 10 minutes at 4° C followed by cell surface antibodies in FACS Buffer (PBS with 2% FBS) for 30 minutes at 4° C. Cells were pelletized at 1500 rpm for 5 minutes before re-suspending in 200 μL of FACS Buffer. Expression of T cell surface markers was measured by fluorescence cytometry

(MACSQuant, Miltenyi Biotec) and analyzed by FlowJo software (Tree Star Inc.).

Statistical Methods.

Graft volumes were compared at the indicated timepoint using a one-tailed T-Test for two-samples with unequal variance (Microsoft Excel). Survival was compared using log-rank (Mantel-Cox) test (GraphPad Prism). Immunohistochemical staining was analyzed by one-way ANOVA with Tukey's test for multiple comparisons, where p-values of <0.05 were considered statistically significant (GraphPad Prism). Flow cytometry comparisons of Control, Flank, and Leg tumors were analyzed using a two-way ANOVA, where p-values of <0.05 were considered statistically significant (GraphPad Prism).

Results

We developed a PD-based iRT approach for CRPC in an immunocompetent castrated syngeneic FVB mouse model using subcutaneous Myc-CaP tumor grafts.[375,377] The expression of PD-L1 was found to be dynamically altered by radiation treatment, depending on the radiation dose administered. The highest expression of PD-L1 in Myc-CaP tumor cells was found to be 10 Gy, compared to 0, 2, and 20 Gy treatments (**Supplementary Fig C.S1**). Thus, this dose was selected for the preclinical tumor graft model. Mice were treated with only 8 days of therapy that included two doses of X-ray radiation treatment combined with either anti-PD-1 or anti-PD-L1 immune checkpoint inhibitor. After the initial treatment responses for both local and distant (unirradiated) tumors during the first 2-3 weeks, the tumors grew and eventually the mice reached the predetermined endpoint. Compared to mice treated with antibody alone, XRT (20 Gy in 2 fractions) to the leg tumor graft causes a local response in the irradiated tumor and a robust

abscopal effect with regression of an unirradiated distant tumor graft (**Figures 4.1A and 4.1B**). At 21 days, the mean graft volume for anti-PD-1 alone was 2094 mm^3 (N=18 grafts) compared to iRT irradiated grafts 726 mm^3 (N=9 grafts) (p=0.04) and unirradiated grafts 343 mm^3 (N=9 grafts) (p=0.0066). At 17 days, the mean graft volume for anti-PD-L1 alone was 1754 mm^3 (N=16 grafts) compared to iRT irradiated grafts 284 mm^3 (N=8 grafts) (p=0.04) and unirradiated grafts 556 mm^3 (N=8 grafts) (p=0.21). No significant differences were observed between the leg and flank graft volumes within each treatment group, so both grafts were included in the antibody alone data. As an expansion of the data shown in **Fig 4.1A** and **Fig 4.1B**, the complete tumor growth curves are shown (**Supplementary Figure C.S2)**.

Remarkably, this combined iRT approach significantly increased survival (**Figures 4.1C and 4.41D**). For anti-PD-L1, the median survival for antibody alone was 13 days (N=8 mice) compared to 30 days (N=8 mice) for iRT (p=0.0003). For anti-PD-1, the median survival for antibody alone was 21 days (N=9 mice) compared to 36 days (N=9 mice) for iRT (p=0.0009).

Similar to clinical data showing lack of efficacy for immune checkpoint monotherapy, we found that mean graft volume for untreated grafts was similar to anti-PD-1 (p=0.19) and anti-PD-L1 (p=0.24) antibody treatment alone, respectively (**Figure 4.2A**). Furthermore, the survival of mice without treatment or those treated with XRT alone were similar (p=N.S.) to those treated with anti-PD-1 alone or anti-PD-L1 alone (**Figure 4.2B**). This indicates the importance of combination treatment over monotherapy in this preclinical model.

Radiation and Immune Checkpoint Blockade Decrease Tumor Burden and Increase Survival

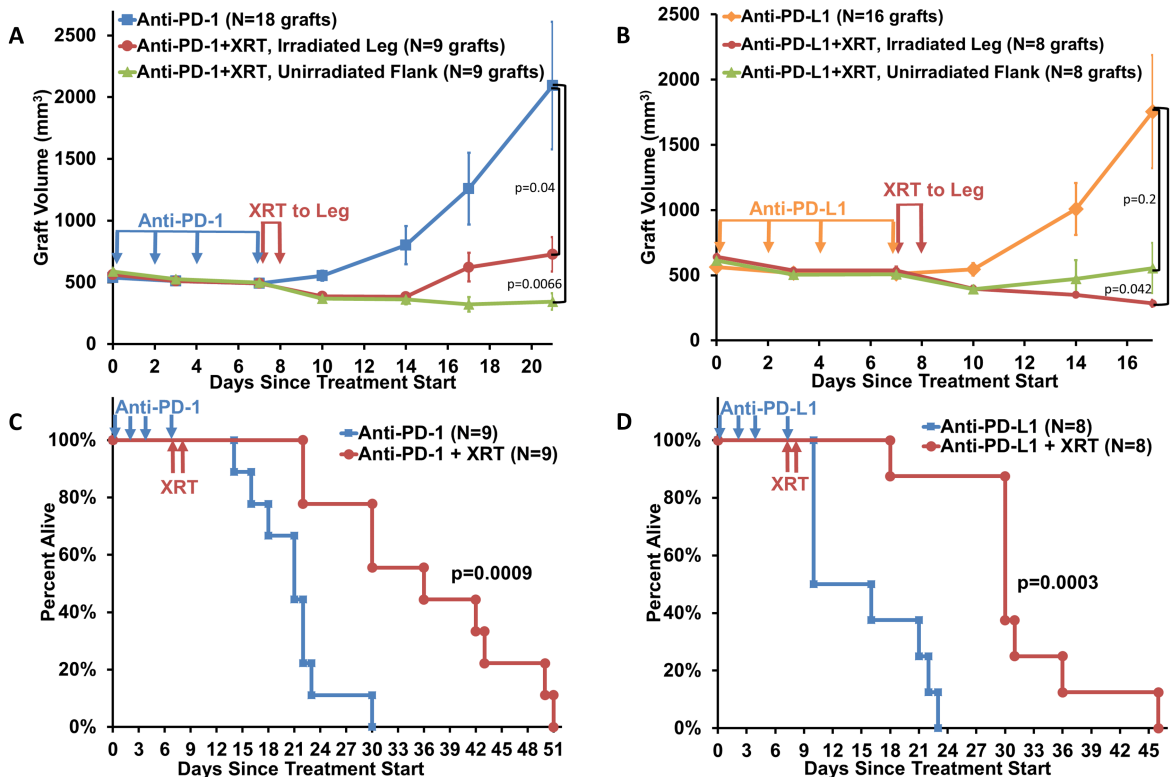

Figure 4.1. Castration-resistant prostate cancer is successfully treated by immune checkpoint inhibitor combined with radiotherapy, with effects on the irradiated and unirradiated tumors, and increased survival. A-D. Myc-CaP tumor graft volumes (A and B) and survival (C and D) for mice treated with immune checkpoint inhibitor monotherapy and given in combination with XRT to the leg graft. Significantly decreased tumor graft volume and significantly increased median survival was observed. Error bars represent ± SEM.

Radiation or Immune Checkpoint Blockade as Monotherapy Did Not Alter Tumor Growth or Survival

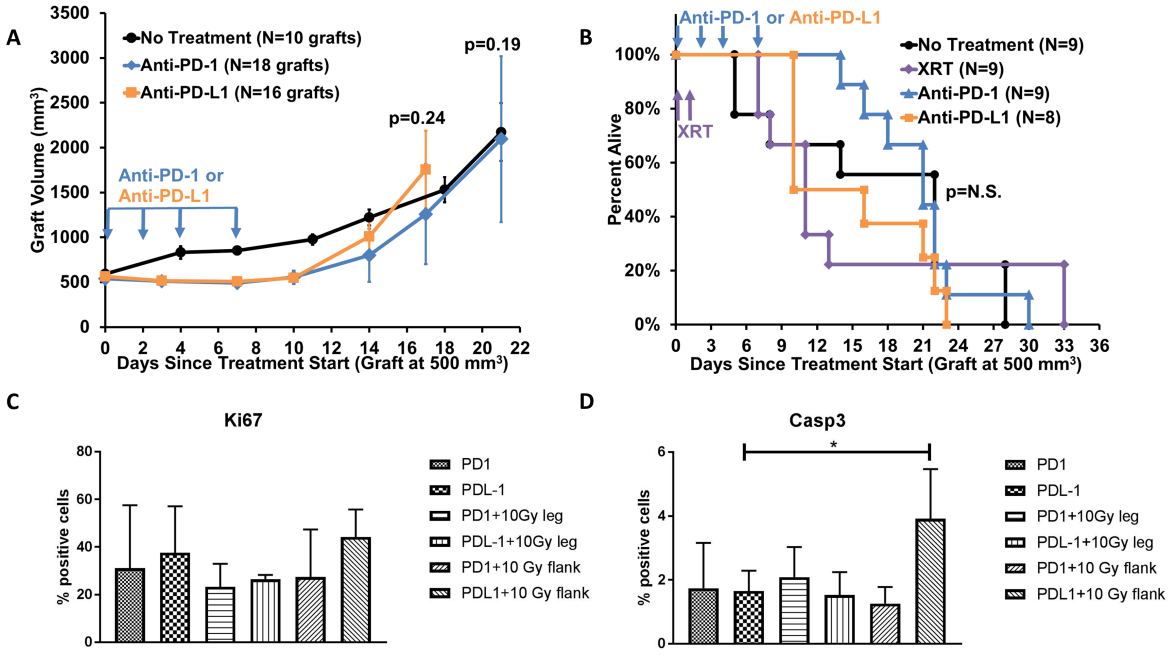

Figure 4.2. Monotherapy is similar to no treatment, and tumor cell proliferation and apoptosis is minimally affected. A) Myc-CaP tumor graft volumes for untreated mice and those treated with immune checkpoint inhibitor monotherapy. B) Survival of mice with Myc-CaP grafts, including no treatment, XRT alone, and immune checkpoint inhibitor monotherapy, as indicated, with no significant differences in median overall survival between the groups (log-rank test, $p>0.05$). C and D) Ki67 and cleaved caspase-3 (Casp3) immunohistochemical staining analyzed by whole slide digital imaging. Independent graft numbers analyzed: anti-PD-1 (N=4), anti-PD-L1 (N=6), anti-PD-1 + XRT leg graft (N=4), anti-PD-L1 + XRT leg graft (N=2), anti-PD-1 + XRT flank unirradiated graft (N=3), anti-PD-L1 + XRT flank unirradiated graft (N=3). Error bars represent ± SEM.

To study treatment-related effects on tumor cell proliferation and apoptosis, immunohistochemical staining was performed on grafts harvested at day 16 after starting immune checkpoint inhibitor treatment. There were no differences among the treatment groups for Ki67 or cleaved caspase-3 staining, except for a significant increase in caspase staining ($p = 0.024$) in the unirradiated flank tumor when analyzing anti-PD-L1 combined with XRT compared to anti-PD-L1 monotherapy (**Figures 4.2C and 4.2D**). The mechanism for this finding is under investigation.

Based on other iRT tumor models, we hypothesized that an immune-related treatment mechanism may be mediated by tumor-infiltrating immune cells, especially CD8+ T cells. The tumor microenvironment was studied by flow cytometry on tumor tissue harvested at day 14-17 after starting immunotherapy, which provides quantification of tumor infiltrating lymphocytes (TILs). After selecting live lymphocytes, appropriate T cells populations were selected using double positive CD8+CD3+ gates or CD4+CD3+ gates, while natural killer (NK) cells were selected as live lymphocytes that are CD335+. There was a greater percent of CD8+CD3+ cytotoxic T cells in the untreated control tumors compared to those treated with anti-PD-L1 and XRT (**Figure 4.3A**). T cells were then analyzed for markers of exhaustion and activation. In mice treated with anti-PD-L1 antibody and XRT to the leg tumor, both flank and leg tumors had significantly higher expression of PD-1 in CD8+CD3+ cytotoxic T cells (**Figure 4.3B**). Additionally, the leg tumor treated with radiation and anti-PD-L1 had higher CD44+ expression on CD8+CD3+ cytotoxic T cells compared to untreated control tumors. (**Figure 4.3C**). CD4+ tumor-infiltrating immune cells were characterized by flow cytometry. Although there was no significant change in the total CD4+ cells within tumor grafts, the subset of PD1+ CD4+ was enriched after treatment with anti-PD-L1 antibody in both the irradiated leg graft and unirradiated flank graft in the same mice (**Supplementary Figure C.S4A, C.S4B)**. In addition, the frequency of T regulatory cells was elevated in both tumors from the irradiated leg and unirradiated flank graft compared to the control mouse tumors (**Supplementary Figure C.S4C**. Furthermore, CD335+ tumor infiltrating CD335+ cells were significantly increased in the grafts treated with anti-PD-L1 and XRT compared to untreated control (**Figure 4.3D**).

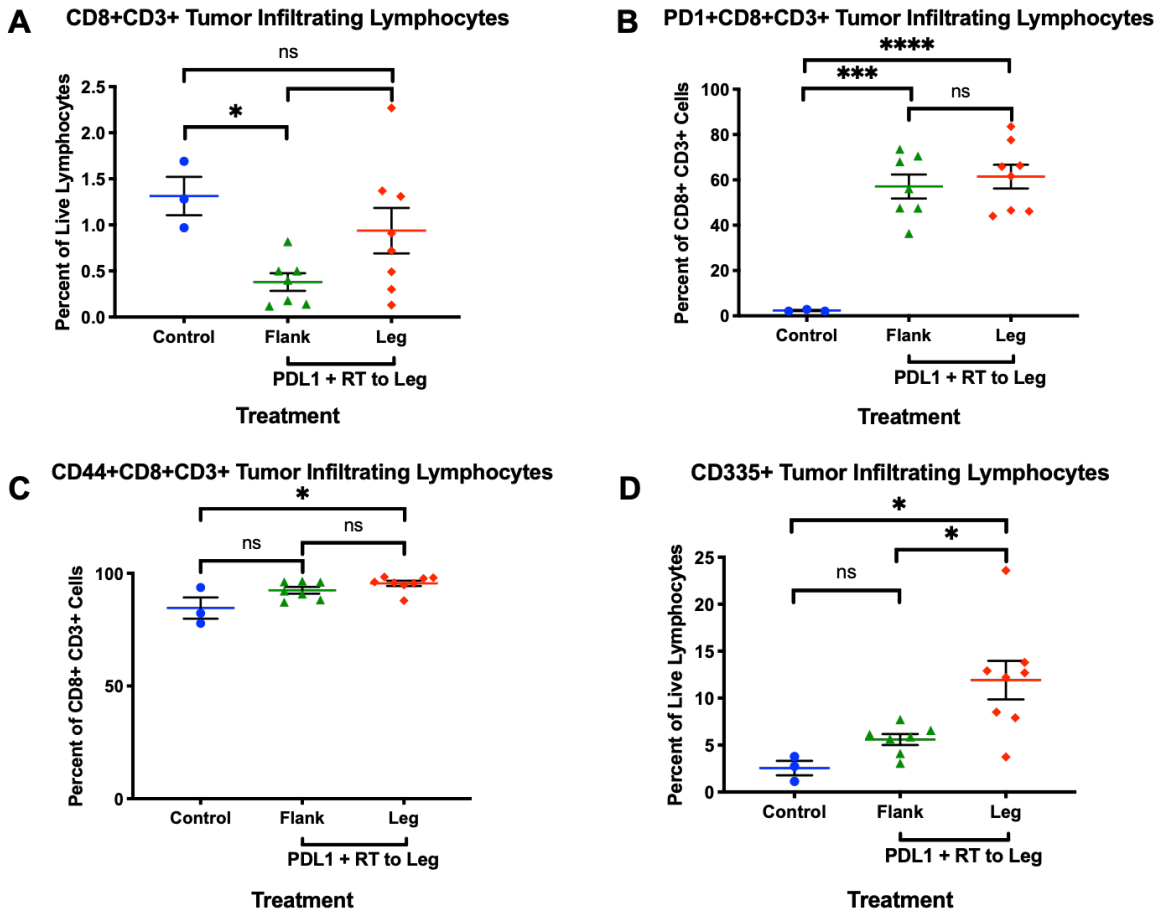

Figure 4.3. Immune profile in tumor microenvironment. A-C. Flow cytometry for double positive CD8+ and CD3+ T cells and expression of selected markers (PD-1 or CD44) on CD8+ T cells within Myc-CaP untreated control tumors (N=3), flank tumors from mice treated with anti-PD-L1 and XRT to leg tumor (N=7), or leg tumors that received direct XRT and ant-PD-L1 treatment (N=8). D. Flow cytometry for CD335+ NK cells from live cells within Myc-CaP untreated control tumors (N=3), flank tumors from mice treated with anti-PD-L1 and XRT to its leg tumor (N=7), or leg tumor that received direct XRT and anti-PD-L1 treatment (N=8). Error bars represent ± SEM; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, and ****$P < 0.0001$, two-way ANOVA test.

The role of CD8+ cells in iRT response was verified in the Myc-CaP CRPC mouse model by depleting CD8+ cells by three once-weekly injections of anti-CD8a antibody.[378] The results show loss of the survival advantage, which suggests that part of this iRT mechanism is mediated by a CD8+ cell (**Figure 4.4A**).

CD8 T Cell Depletion Inhibited Survival Increase from Immunotherapy and Radiation Treatment

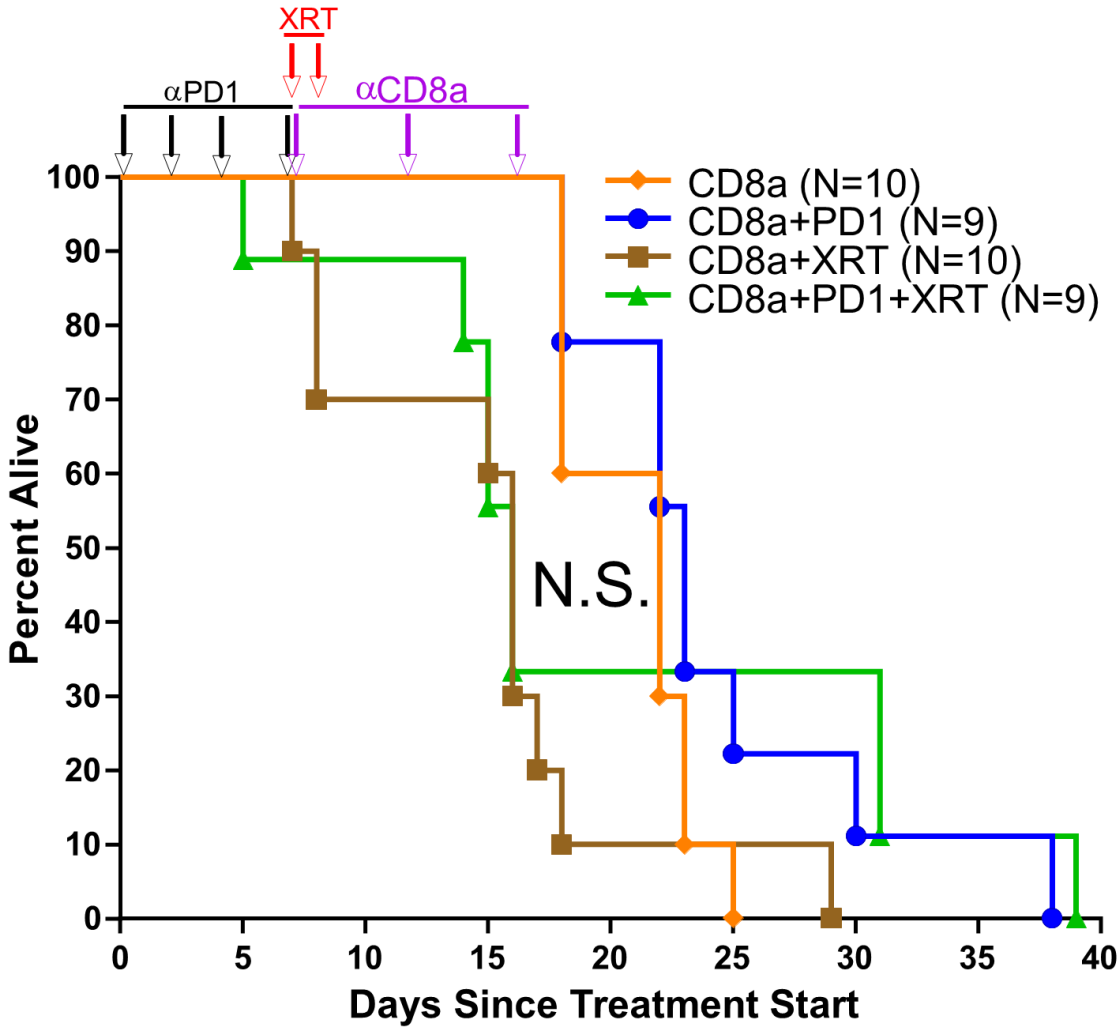

Figure 4.4. CD8 depletion blocks survival advantage from combination radiotherapy and immune checkpoint inhibition. Survival for mice with Myc-CaP tumor grafts treated with anti-CD8a antibody to deplete CD8+ cells. Mice received no additional treatment, anti-PD-1 immune checkpoint inhibitor alone, XRT alone, or anti-PD-1 in combination with XRT to the leg graft. N.S. = non-significant median survival difference.

Discussion

Although clinical data suggests limited effects of immune checkpoint inhibitor treatment for CRPC, this preclinical model indicates robust responses are achievable using when combining anti-PD-1 or anti-PD-L1 treatment with XRT. A syngeneic mouse model was selected to allow treatment effects to be studied in the presence of an intact immune system. The highly-aggressive Myc-CaP model in the castration-resistant setting was selected to investigate treatment efficacy.

Tumor graft growth was significantly diminished by the combination treatment of immune checkpoint inhibitor and XRT compared to drug alone. Remarkably, unirradiated distant tumor grafts also responded to combination treatment, suggesting an abscopal treatment effect. Most importantly, significant increases in median survival were observed compared to antibody treatment alone: 70% longer for anti-PD-1 and 130% for anti-PD-L1. Importantly, no increased toxicity was observed for combination immuno-radiotherapy treatment compared to monotherapy. However, a notable limitation of this preclinical model is that the combination treatment was not found to be durable after a single treatment cycle (8 days), with no mice completely clearing their tumor grafts. It is possible that repeat dosing by immune checkpoint inhibitor would extend the treatment effect, as found in clinical studies using immune checkpoint inhibitors, but this was not investigated in this preclinical model. Furthermore, additional treatment combinations are currently being tested to determine the best approach, including varying the timing/sequencing of therapies and the radiation dose/fractionation.

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To further understand the mechanism for decrease in tumor growth resulting from combination XRT and anti-PD-L1 antibody treatment, flow cytometry was used to characterize the tumor immune microenvironment. When analyzing only live cells, there was a higher percentage of CD8+ cytotoxic T cells in the tumors of control mice compared to flank tumors from mice that received systemic anti-PD-L1 antibody treatment and radiation treatment to the leg tumors. However, flow cytometry showed strong differences in activation between the tumor infiltrating lymphocytes in the control group compared to treated mice. Both flank and leg tumors from treated mice had significantly more CD8+ cytotoxic tumor infiltrating T cells expressing PD-1. Additionally, the XRT-treated leg tumors showed a significantly higher percentage of CD8+ cytotoxic T cells expressing CD44, a marker of T cells that are active after antigen presentation. Although the decreased T cell infiltration in treated tumors does not indicate a mechanism for decreased tumor

growth in mice treated with radiation and anti-PD-L1 antibodies, the differences in activation can potentially account for these differences. The increased expression of both PD-1 and CD44 suggests that the tumors from mice treated with radiation and anti-PD-L1 are experiencing increased rates of tumor antigen presentation, which could be one mechanism for decreased tumor growth in the treated mice. It is also possible that CD335+ NK cells play a role in the tumor microenvironment, as supported by the flow cytometry data indicating an increase in the mice treated with anti-PD-L1 and XRT. Lastly, the survival advantage is lost when blocking CD8 in the mice, suggesting a key mechanistic role for CD8+ cells in the immune response. Additional mechanistic roles of the immune cells are being investigated, since the immunity triggered by combination immune checkpoint and radiotherapy is complex.[379]

Emerging clinical data indicates about 3% of patients with prostate cancer have a high tumor mutation burden (microsatellite instability-high or mismatch repair deficit) and they are responsive to anti-PD-1/PD-L1 agents, with 45% (5 of 11 patients) experiencing durable clinical benefit.[380] National Comprehensive Cancer Network guidelines for metastatic CRPC include consideration of testing tumor mutation burden and second-line treatment by pembrolizumab. As clinical trials develop to test PD-agents for prostate cancer treatment, it is important to recognize that an immune checkpoint treatment combined with radiotherapy may provide an even greater response rate than monotherapy. The preclinical model presented herein provides a framework for further investigating the optimal approach for combining radiotherapy and PD-agent that can be carried into future clinical trials.

V. CONCLUSIONS

Chapter Summaries and Impact

The overall goal of my dissertation project was to identify how obesity affected cancer immunotherapy efficacy in a mouse model. When I started this project, there were no published papers to date investigating obesity and immunotherapy in mice. Therefore, I overcame a significant number of hurdles optimizing the mouse model including choosing the best mouse strain, compatible cell line, and immune-checkpoint blockade dosing to ensure reproducibility. In the first aim of this dissertation (Chapter II), I hypothesized that obese and lean mice would have different immunotherapy efficacy due to the macrophage changes in obesity. Chronic systemic inflammation in obesity over long periods leads to subsequent increases in MDSCs in the adipose tissue, spleen, and other tissues such as tumors.[156] These MDSCs then secrete IL-10, supporting a M2 macrophage polarization of TAMs. The TAMs are anti-inflammatory and pro-tumor, secreting a variety of cytokines and growth factors that support tumor growth and lead to worse outcomes in patients.[140,381,382]

In this chapter, I first characterized immune differences between obese and lean C57BL/6 non-tumor bearing mice. T cells from obese mice had increased frequencies of PD1 and CD44 expressing cells compared to lean mice T cells. These findings support the chronic inflammation model in obesity, where the T cells have undergone antigen presentation and subsequently upregulated CD44 and PD1. When MC38-CEA1 tumors were subcutaneously injected in obese and lean C57BL/6 mice, tumors grew significantly larger in the obese mice. In the tumor-bearing mouse spleens, the obese mice had lower T cell frequencies but increased CD44 and PD1 compared to lean mice. There were no

significant differences in the $CD8^+$ tumor-infiltrating lymphocytes, but the tumor-associated macrophages in lean mice had stronger M1 polarization determined by iNOS2 production. When treating the obese and lean MC38-CEA1 tumor bearing mice with αPD-1 antibodies, both obese and lean mice responded to treatments, but obese mice had a larger decrease in tumor volume. However, the proportion of volume change between obese IgG versus anti-PD-1 treated mice and lean IgG versus αPD-1 treated mice was not different. There were no significant differences in tumor-infiltrating lymphocytes between obese and lean mice or between IgG and αPD-1 treated mice, except for significant knockdown of PD-1 expression on T cells in the αPD-1 treated groups. The only significant differences measured in the tumor microenvironment were changes in TAM polarization. In the IgG treated groups, the lean mice had increased frequency of MHCII expressing TAMs compared to obese mice. When the obese mice were treated with αPD-1 antibodies, the TAMs repolarized to an M1 frequency that matched that of lean mouse TAMs. There was no significant difference in M1 polarization between lean IgG and αPD-1 treated mice. Altogether, my first aim determined that obese mice treated αPD-1 antibodies experienced tumor-associated macrophage polarization to an M1 phenotype unique for obese mice, which subsequently led to a larger decrease in tumor volume in obese mice compared than lean mice. These findings support future studies exploring the roles of macrophages in immunotherapy efficacy and targeting macrophage polarization to enhance immune checkpoint blockade.

The second aim of this work (Chapter III), hypothesized that exogenous leptin would increase M1 macrophage polarization, decrease tumor growth, and enhance immunotherapy efficacy. Adipose tissue is the primary producer of leptin, and as adipose tissue accumulates in obesity, leptin levels subsequently also increase. Leptin directly

increases M1 polarization of macrophages. Given that leptin promotes Th1 T cell phenotypes and cytotoxic $CD8^+$ T cells, leptin increases IFNγ production, indirectly supporting an M1 phenotype. To simulate elevated leptin levels that occur in obesity, lean mice were treated with leptin injections twice a day for two weeks before injecting MC38-CEA1 tumor cells. When treating lean MC38-CEA tumor-bearing mice with exogenous leptin, splenic macrophages and tumor-associated macrophages had a greater M1 phenotype than control mice, indicated by increased expression of CD86 and MHCII. Additionally, splenic $CD8^+$ T cells from leptin-treated mice had increased expression of PD1 and CD44 compared to controls, which is the same pattern that was seen in spleens from obese mice compared to lean mice. When combining immunotherapy with leptin treatments, an acute leptin treatment model starting the injections simultaneously with αPD-1 antibodies was used to determine if leptin could be used to enhance immunotherapy efficacy by increasing M1 TAM polarization. The mice treated with both leptin and αPD-1 had the smallest tumors. Co-treated tumors have the lowest frequency of tumor-associated macrophages and the greatest M1 polarization by MHCII and iNOS2 expression compared to control, leptin, or αPD-1 treated mouse tumors. Together, this chapter identified that macrophage repolarization by leptin is a successful method for enhancing immunotherapy efficacy.

The third aim of this work (Chapter IV) focuses on enhancing immunotherapy efficacy with radiation treatment. Clinically, radiation treatment has induced an abscopal effect, where a tumor regression occurs in a non-irradiated site. Although this effect is unpredictable and poorly understood, it suggests that immune activation can occur with radiation treatment. The co-treatments have the potential to increase the effects of immunotherapy and increase the chances of inducing an abscopal effect. For these studies,

we used a castration-resistant prostate cancer mouse model that is poorly responsive to immunotherapy treatments when given as a monotherapy. Castrated FBV mice received subcutaneous Myc-CaP prostate cancer cell grafts in both the leg and flank. For the combination treatment experimental groups, mice receive either anti-PD-1 or anti-PD-L1 antibodies systemically and each leg graft received 20 Gy in 2 fractions. Mice that received co-treatments of immunotherapy and radiation had a significant increase in survival compared to mice treated with immunotherapy alone, radiation alone, or control-treated mice. This increase in survival did not occur when depleting $CD8^+$ T cells from co-treated mice, suggesting that the $CD8^+$ T cells are required for the decrease in tumor growth. An abscopal effect occurred in mice receiving the co-treatment radiation and immunotherapy, where the non-irradiated tumors had similar growth curves to radiated tumors. The work detailed in Chapter IV demonstrates that both increased survival and an abscopal effect can be achieved by co-treating mice with immunotherapy and radiation. These findings are important for supporting clinical trials that will test and identify the best protocols for combining immune checkpoint blockade and radiation in patient care.

This work has contributed to our understanding of immunotherapy efficacy and how we can enhance immunotherapy efficacy in patients. I have demonstrated that the macrophages play a role in decreasing tumor growth with immunotherapy treatment, particularly in obesity. Through the work in chapter III and IV, I have identified that macrophage repolarization via leptin or radiation can improve immunotherapy efficacy. These findings open the doors for a breadth of preclinical and clinical studies to further examine macrophages and radiation biology in the setting of immune checkpoint blockade. My work identifies potential clinical strategies for improving immunotherapy efficacy and increasing cancer patient survival.

Shortcomings

There are always shortcomings when using preclinical models to help identify mechanisms of drug responses in patients. When designing preclinical studies consistency and reproducibility is always a goal of the experimental design. For this reason, we chose the C57BL/6 mouse model on a 60 kcal diet, which is the most commonly DIO model used.[383] Despite the same genetics and diet, not all of the DIO mice gained the same amount of weight, resulting in up to a 15 gram difference in weights by the end of the experiments. This model has even greater shortcomings when beginning to compare it to humans. In patients, there is a wide variety of other obesity components that effect tumor growth and microenvironment that are not captured by our modeling including dietary intake and microbiome, which effect systemic inflammation.[384] Specifically, the microbiome can influence immunotherapy efficacy in mice and patients, and the mouse microbiome varies between housing environments, even within the same institution.[385–387]

Additionally, the location of the tumor as subcutaneous versus orthotopic significantly influences the types of immune cells in the tumors. For an orthotopic model of colon cancer, intracolonic injections can be performed, but these procedures are technically difficult, labor intensive and have low success rates.[388] Due to the increased cost and time for doubling the number of mice for experiments and the inability to perform intracolonic injections in the mouse barrier facility, our studies elected to use subcutaneous injections. Subcutaneous tumors of CT26 colon cancer cells in balb/c mice have more myeloid-derived suppressor cells and fewer T cell, B cells, and natural killer cells compared to orthotopic CT26 tumors.[389] Orthotopic CT26 tumors also had an increase in pro-inflammatory cytokines, IL-2, IL-6, IFNγ, and granzyme B and a decrease in

expression of checkpoints PD-1, PD-L1, and CTLA-4 compared to subcutaneous tumors.[389] Subsequently, orthotopic CT26 tumors treated with anti-PD-1 and anti-CTLA-4 all regressed completely, while subcutaneous CT26 had decreased tumor volume and only one tumor completely regressed.[389] The tumor immune microenvironment in orthotopic tumors has greater potential to generate anti-tumor responses after immune checkpoint blockade treatment. Given the differences in immune infiltrates of subcutaneous and visceral adipose tissues in obesity, orthotopic models would better model the effects of peritumoral adipose tissue on tumor growth and immune checkpoint blockade efficacy.[41,59]

The descriptions of M1 and M2 macrophages are useful for general descriptions of macrophage function, and these labels apply well to polarized macrophages *in vitro*.[350] Macrophage function and receptor expression *in vivo* can overlap between both phenotypes, which is why macrophages are often described as being on a spectrum.[351,352] Additionally, the M1/M2 macrophage spectrum and function are substantially different in murine versus human macrophages, and gene profile suggest that only 50% of genes are shared between mouse and human macrophages.[390] Therefore, our studies investigating macrophage repolarization need to be investigated in human tumors, preferably with pre-treatment and post-treatment biopsies or resections.

A final notable shortcoming is the length of time required to process biological samples for immunological assays. Experiments for my dissertation project initially used 12 mice total, where only the tumors were collected and processed into single cell suspensions in a few hours. However, as the experiments expanded to 40 mice and the number of tissues collected increased to include tumors, spleens, serum, adipose tissue, and livers, the amount of time spent processing the tumors and spleens into single cell suspensions exponentially increased. While all cautionary steps were taken to ensure

maximum viability of cells, no testing was performed to determine which cell types had the best viability. For example, if many tumor cells died in comparison to immune cells, then the frequency of CD45 cells amongst live cells would be inflated. Differences in viability of immune cell subtypes of T cells versus macrophages and myeloid-derived suppressor cells could also create an inaccurate depiction of live cell frequencies in the tumors. Despite these shortcomings, the experiments conducted used appropriate models to achieve the goals of the dissertation research.

Future Work and Potential Applications

Future work investigating the effects of obesity on immunotherapy efficacy and methods of enhancing immune checkpoint blockade need to use more translational models that better simulate human immune system and tumor interactions. The C57BL/6 mouse model is the most widely used diet-induced obese preclinical model, and it accurately portrays metabolic syndrome and diabetes that can develop in obesity and increase cancer progression.[383,391] Obese patients with cancer often present with more advanced disease stages and with greater disease burden than lean patients, which mirrors the increase tumor size at time of treatment for the DIO mice compared to lean mice.[392] However, despite the best research efforts to use representative, reproducible preclinical models, the heterogeneity that exists in patients is inadequately reflected in most mouse models. These heterogeneities in patients include tumor heterogeneity, dietary differences, and microbiome species, which can impact immunotherapy efficacy.[384] To address tumor heterogeneity, DIO studies could be conducted in mouse models with spontaneously arising tumors such as the MMTV-PyMT transgenic breast cancer model or in genetically engineered mouse (GEM) models. Before the immunotherapy era, the majority of

transgenic and GEM tumors were not immunoresponsive, but more immunoresponsive models are becoming available, such as the YUMM melanoma cell lines, to perform immunotherapy experiments in sporadic tumor mouse models.[393] To examine the effects of dietary fats on immunotherapy efficacy, a variety of diets concentrated in different types of fat could be used to including inflammatory acids (saturated fats, Ex: lauric and stearic acid) or anti-inflammatory fats (monounsaturated fatty acids, Ex: palmitoleic acid and oleic acid). This study would be particularly useful as some pro-inflammatory fats have been linked to an increased risk of colon carcinogenesis, but the increase in inflammation may be useful in an immunotherapy treatment.[123] Initially murine studies identified that *Bifidobacteria* in the microbiome were associated with a response to immunotherapy.[386] Follow-up studies in patients identified that immunotherapy responders had higher concentrations of *Bifidobacterium longum, Collinsella aerofaciens*, and *Enterococcus faecium* in their gut microbiome.[387] While obese people have increased levels of *Collinsella aerofaciens* in their gut microbiome, studies have shown that taking *Bifidobacterium* and *Enterococcus faecium* as probiotics can result in weight loss.[394–396] Therefore, microbiome alone is not the sole mechanism for increased immunotherapy efficacy in obesity. To conduct informative microbiome and immunotherapy studies, I would identify the microbiomes in the lean and DIO mice before and after immune checkpoint blockade treatments. Then, DIO microbiome transplants would be conducted in lean mice before initiating further immunotherapy experiments to identify if the DIO mouse microbiome enhances immunotherapy efficacy or affects macrophage polarization in lean mice. Additionally, mice in different housing facilities have different microbiomes.[397] Because my mouse experiments were conducted in the specific pathogen free barrier facility, I would expect that the mouse microbiomes from my mice would be

less diverse than if the mice were housed in standard mouse facility. These examples are just a few limitations for mouse models, and future studies should develop methods to address these translational shortcomings in mouse models to improve translational impact.

While obese mice had a larger decrease in tumor volume with immunotherapy treatment, the proportion of tumor volume decrease between obese and lean mice treated with immunotherapy versus IgG antibody was not different. Given the differences in initial tumor size between obese and lean mice in the DIO models, future experiments will need to investigate whether the increased immunotherapy efficacy in obesity is influenced by differences in initial tumor burden and tumor immune infiltration. To determine if macrophages are required for enhanced immunotherapy efficacy in obesity, DIO immunotherapy studies with macrophage-depleted mice via clodronate or CSF1R antibodies should be conducted. Similarly, to determine if leptin is required for the macrophage repolarization with immunotherapy, leptin-blocking antibodies could be used during immunotherapy experiments.

Now that the macrophages have been identified as a major player in immunotherapy studies, further targeted-macrophage manipulation should be tested. The mannose-decorated nanoparticles (Man-NPs) from Dr. Giorgio's lab that target macrophages via the mannose receptor are the perfect tool for macrophage manipulation. Classically, the Giorgio lab has encapsulated IκBα siRNA. The siRNA delivery prevents IκBα from inhibiting the classical M1 macrophage activation pathway and NF-κB activity, thereby increasing the M1 polarization of macrophages. These Man-NPs would provide a biocompatible and targeted method to manipulate TAMs without affecting other immune

cells, unlike the systemic leptin injections. First, the Man-NPs would treat lean and obese mice alone to determine how macrophage polarization would affect tumor growth at baseline. Then, obese and lean mice would be co-treated with αPD-1 and Man-NPs, along with the appropriate controls and monotherapies, to determine if targeted polarization of TAMs will enhance immunotherapy efficacy in both obese and lean mice. With the large number of control nanoparticle and dosing optimization experiments needed, this would be an excellent area of study for a future graduate student.

MC38 proliferation was not increased in the presence of leptin, presumably due to the absence of leptin receptors on MC38 cells in this model. Other cancer types such as some breast cancers do express a leptin receptor, and future studies should investigate the effect of leptin on tumor and immune cells in immunotherapy models.[398] Previous studies with orthotopic melanoma tumors have demonstrated that obese mice have higher PD-1 expression and dysfunction in T cells throughout multiple organs and tumors compared to lean mice, but this was not seen in our model.[214] Given that myeloid cells are more prominent in subcutaneous tumors and T cells are more frequent in orthotopic tumors, immunotherapy experiments with orthotopic intracolonic tumors in DIO mice would assist in understanding tumor immune cell differences between DIO immunotherapy models.[389] To enhance translational potential, clinical studies comparing pre-treatment tumor specimens and post-treatment changes are also of utmost importance to determine if there is a correlation between macrophage frequency and polarization with immunotherapy efficacy.

Identifying that radiation treatment enhances immunotherapy efficacy and increases the abscopal effect has promising implications for additional mechanistic studies and

clinical trials. The greatest clinical challenge for combining radiation and immunotherapy treatments will be determining the timing of each treatment for the greatest immune response and abscopal effect, but without significantly increased rates of immune related adverse events. Additional murine studies should test if radiation should be used as a neoadjuvant, adjuvant, or follow-up treatment with immune checkpoint blockade. While radiation increases the infiltration of anti-tumor immune cells and functions such as $CD8^+$ T and DC cells, MHC expression, and IFNγ production in some murine tumor models, radiation can also increase immunosuppressive cells including T regulatory cells, myeloid-derived suppressor cells, and M2-like TAMs in other murine tumor models.[399] Our studies focused on T cells and natural killer cells, but additional studies analyzing the macrophage and myeloid-derived suppressor cells changes are needed. Radiation also effects the tumor stroma, such as decreasing vasculature and stiffening the extracellular matrix.[399] Therefore, if radiation was used as a neoadjuvant with immunotherapy, the αPD-1 antibodies may not be able to enter the tissue vasculature as well, resulting in decreased immunotherapy efficacy. Meanwhile, pneumonitis is an immune related adverse event that can occur in radiation or immunotherapy treatment. When combining these treatments, clinical trial studies need to ensure that an increase in immune related adverse events does not occur. These potential clinical problems support the need for additional pre-clinical murine studies to optimize treatment timing and further identify immune cell changes in mice treated with radiation and immune checkpoint blockade.

Conclusion

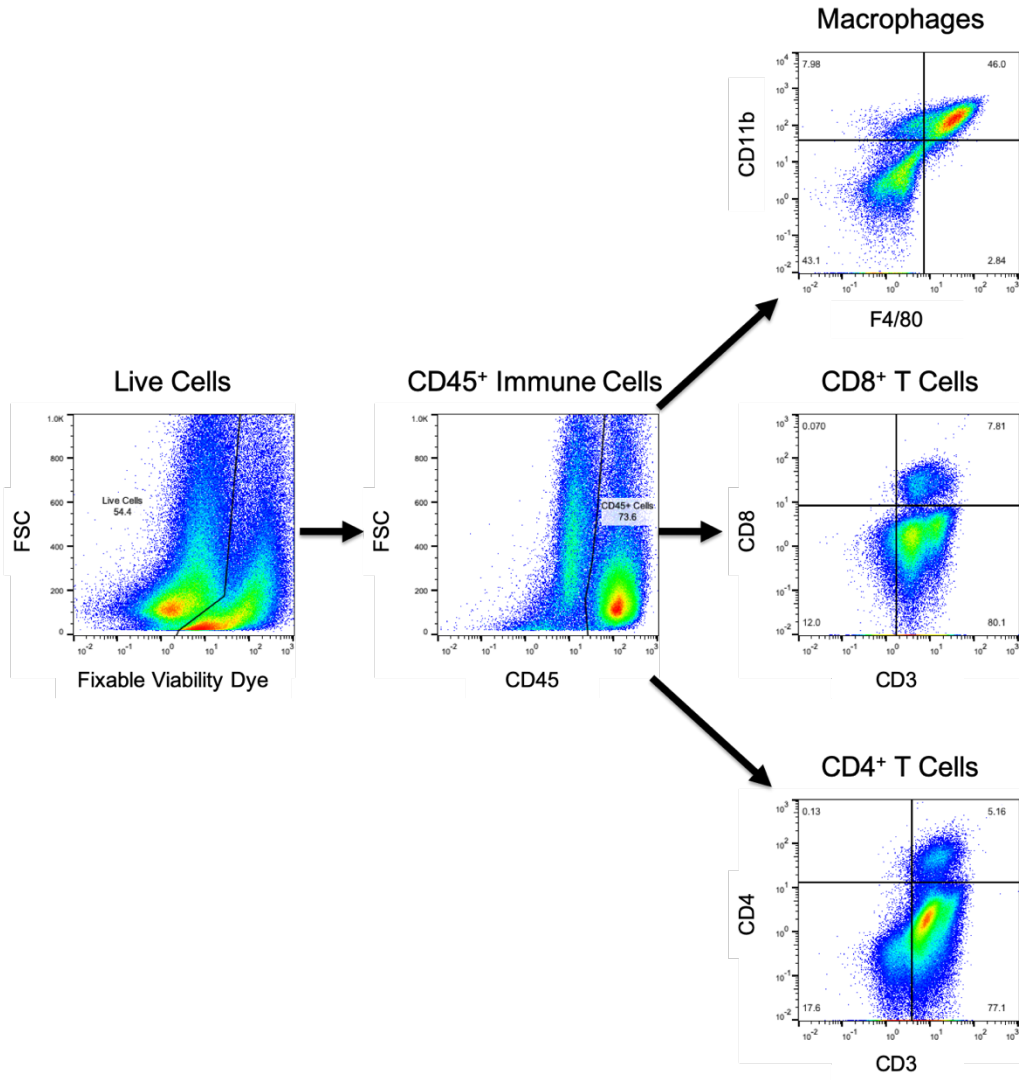
In 2011, the FDA approved the first immune checkpoint blockade antibody for the treatment of melanoma. Less than a decade later, immune checkpoint blockade treatments are approved for 14 different types of cancer. While the patients eligible to receive this potentially life-saving treatment has greatly expanded, our understanding of who responds best to treatment is still poorly understood.

The work described in this dissertation focuses on how obesity affects immunotherapy efficacy and mechanisms to increase immunotherapy efficacy. I identified that obese mice respond better to immunotherapy treatment than lean mice. The studies highlight the importance of macrophage polarization in immunotherapy efficacy and identify that leptin induces M1 macrophage repolarization, promoting response to immune checkpoint blockade treatment. Last, I identified that radiation is capable of increasing immunotherapy efficacy and enhancing the elusive abscopal effect. From obesity as a general risk factor to the manipulation of macrophage polarization with leptin to applying radiation as a co-treatment, each component of this thesis increases our understanding of how immune cells, particularly macrophages, respond to immune checkpoint blockade treatment and how immunotherapy efficacy can be enhanced. Predicting who will respond to immunotherapy treatment and identifying mechanisms to increase immunotherapy efficacy will ultimately enhance immunotherapy to be a life-saving treatment not just for a minority of patients but potentially for all patients.

APPENDIX A

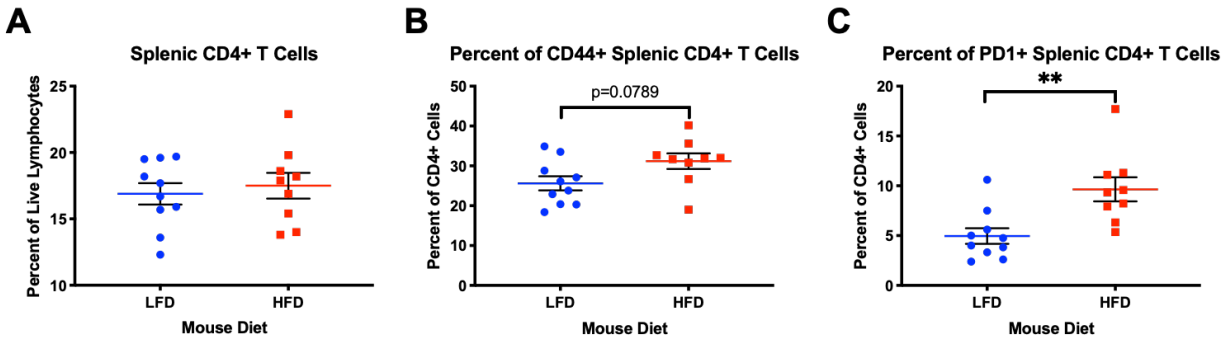
SUPPLEMENTARY MATERIAL FOR CHAPTER II

Flow Cytometry Gating Strategy



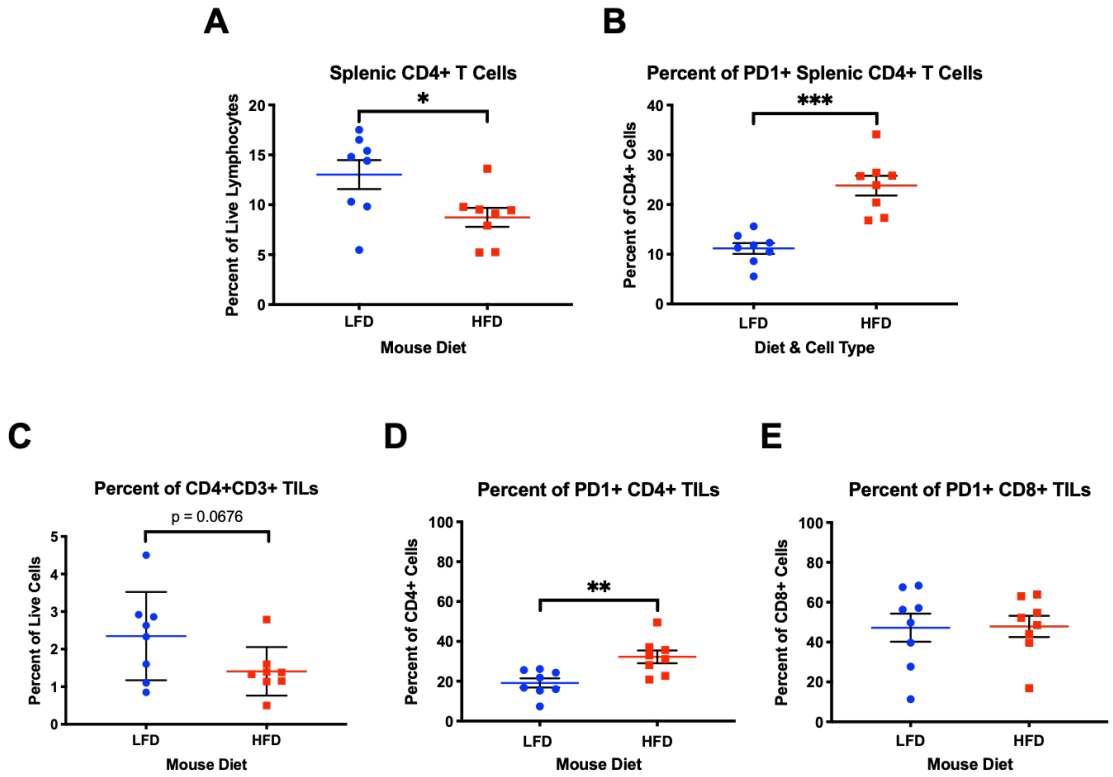
Supplemental Figure A.S1 To identify immune cell populations, a thorough flow cytometry gating strategy was used. First, live cells were selected as those that stained negatively with the fixable viability dye. Then, immune live cells were selected as those positive for CD45+. Last, different immune cells were identified by selecting macrophages as CD11b and F4/80 double positive cells, cytotoxic cells as CD8 and CD3 double positive cells, and helper/T regulatory cells as CD4 and Cd3 double positive cells.

Obesity Induces CD4$^+$ T Cell Exhaustion



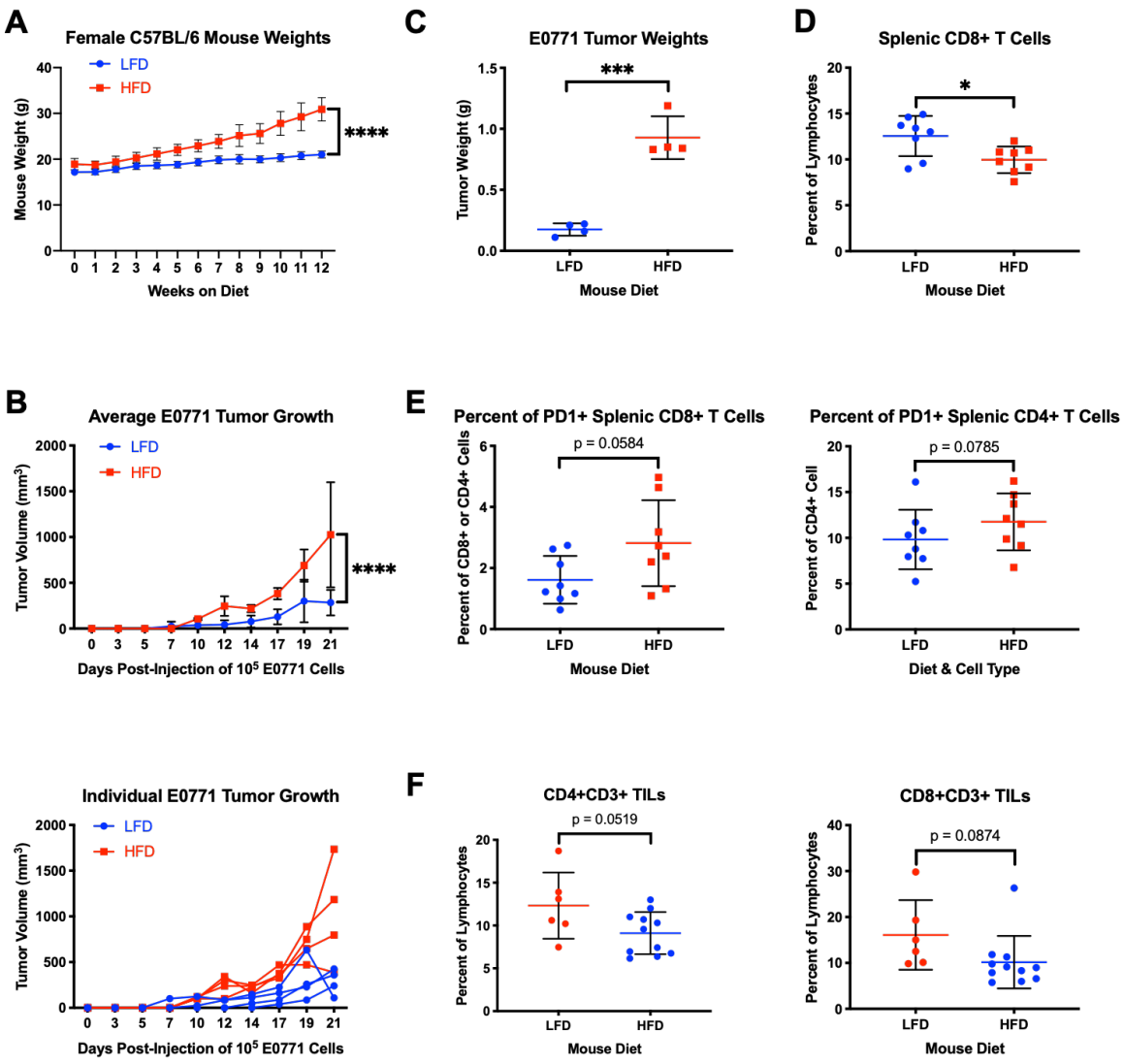
Supplemental Figure A.S2 Five-week old C57BL/6 male mice were maintained on a control standard chow diet (n=8) or 60kcal high-fat diet (n=8) for 12 weeks and were weighed weekly. Two-way ANOVA with Tukey post-hoc test *p* values used. (A-C) Spleens from mice on their respective diet for 12 weeks were processed into single cell suspensions and were analyzed by flow cytometry for cytotoxic T cells. Representative flow plots and frequency from low-fat diet (LFD) (n=10) and high-fat diet (HFD) (n=9) mouse spleens. (A) Frequency of CD4$^+$ splenic T cells. (B-C) Frequency of CD44$^+$ and PD1$^+$ splenic CD4$^+$ T cells. Two-tailed Mann Whitney test *p* values shown. *$p < 0.05$; **$p < 0.01$.

Tumor-Bearing DIO Mice Have Decreased CD4$^+$ T cells, but not CD8$^+$ TILs



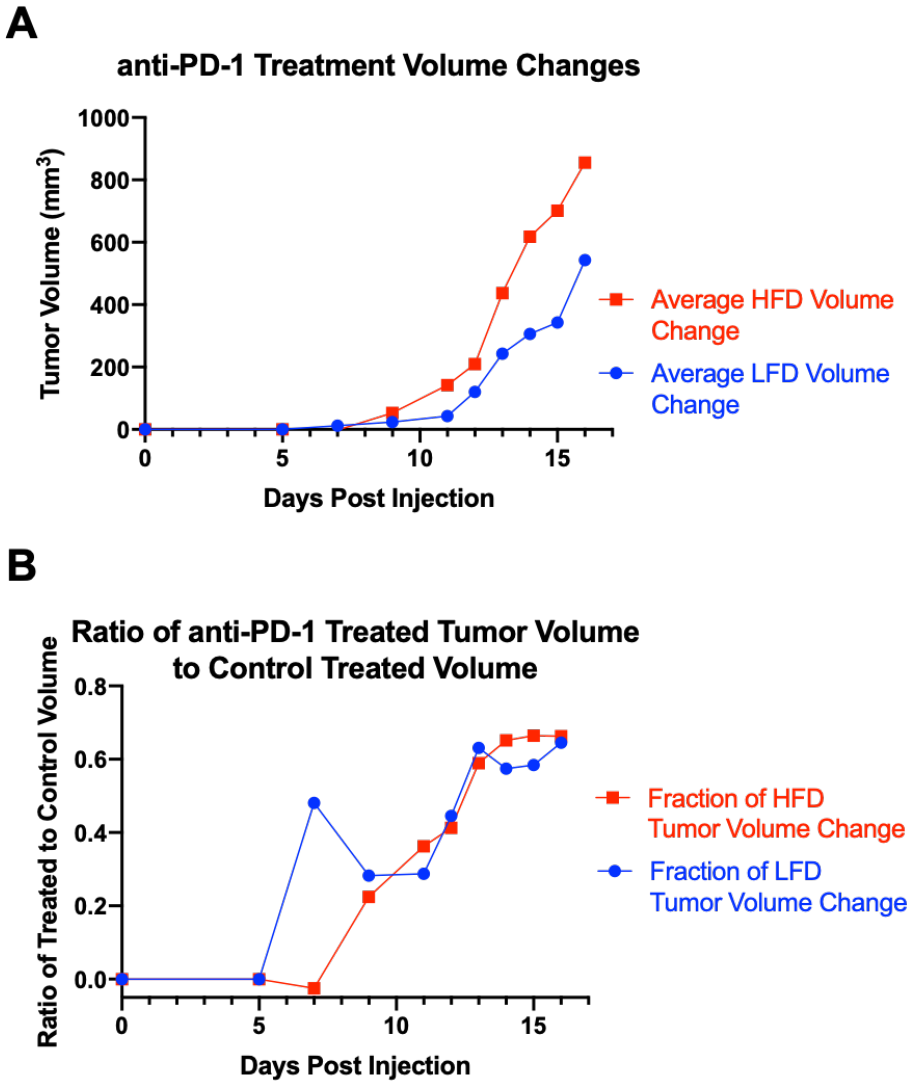
Supplemental Figure A.S3 C57BL/6 male mice on a control standard chow diet (n=10) or 60kcal high-fat diet (n=10) for 12 weeks were injected with 10^5 MC38-CEA1 cells in the right flank. Two-way ANOVA with Tukey post-hoc test *p* values used. (A, B) Spleens from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry. Frequency from low-fat diet (LFD) (n=8) and high-fat diet (HFD) (n=8) mouse spleens. (A) Frequency of CD4$^+$ splenic T cells. (B) Frequency of PD1$^+$ splenic CD4$^+$ T cells. (C-E) Tumors from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry with LFD tumors (n=8) and HFD tumors (n=8). (C) Frequency of CD4$^+$ TILs. (D) Frequency of PD1$^+$ CD4$^+$ TILs. (E) Frequency of PD1$^+$ CD8$^+$ TILs. Data in this figure are all depicted as mean± S.E.M., with all individual points shown. Two-tailed Mann Whitney test *p* values shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

DIO Female Mice with E0771 Orthotopic Breast Tumors Have Similar Immune Profile to MC38-CEA DIO Model



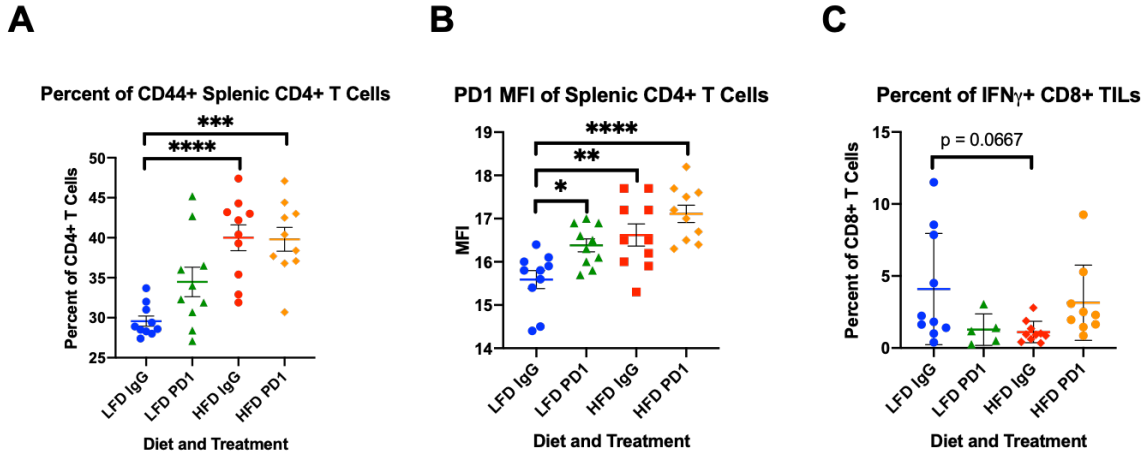
Supplemental Figure A.S4 C57BL/6 female mice on a control standard chow diet (n=8) or 60kcal high-fat diet (n=8) for 12 weeks were injected with 10^5 E0771 breast cancer cells bilaterally in the inguinal mammary fat pads. Two-way ANOVA with Tukey post-hoc test *p* values used. (A) Female mice on HFD weighed significantly more than LFD mice. (B, C) Tumors in HFD mice were significantly larger and heavier than tumors in LFD mice. Spleens from mice with E0771 tumors were collected 21 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry. (D) HFD mice had significantly fewer CD8+ splenic T cells than LFD spleens. (E) Trends in both CD8+ and CD4+ T cells suggested increased PD1 expression on HFD spleens compared to LFD spleens. Tumors from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry. (F) There was a trend of decreased CD4+ and CD8+ TILs in HFD E0771 tumors compared to LFD E0771 tumors. Two-tailed Mann Whitney test *p* values shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

Immunotherapy Induces Similar Decrease in Proportion of Tumor Volume in Obese and Lean Mice



Supplemental Figure A.S5 C57BL/6 male mice on a control standard chow diet (n=10) or 60kcal high-fat diet (n=10) for 12 weeks were injected with 2.5 x 10^5 MC38-CEA1 cells in the right flank. On day 5 post tumor-injection, mice were injected with either 200 µg IgG control antibody or anti-PD-1 antibody, and the injections continued every two days until tumors were collected on day 16 post-injection. (A) The average tumor volume of anti-PD-1 antibody treated mice was subtracted from the average tumor volume of control IgG treated mice. The HFD mice had a larger change in tumor volume compared to the LFD mice. (B) The proportion of anti-PD-1 treated tumor volume to control IgG treated tumor volume is analyzed as the tumors grow. There is no difference in the proportion of treated to control tumor volumes between HFD and LFD mice.

Immunotherapy Does Not Affect Splenic CD4$^+$ T cell Exhaustion or CD8$^+$ TIL Function in Obese or Lean Mice

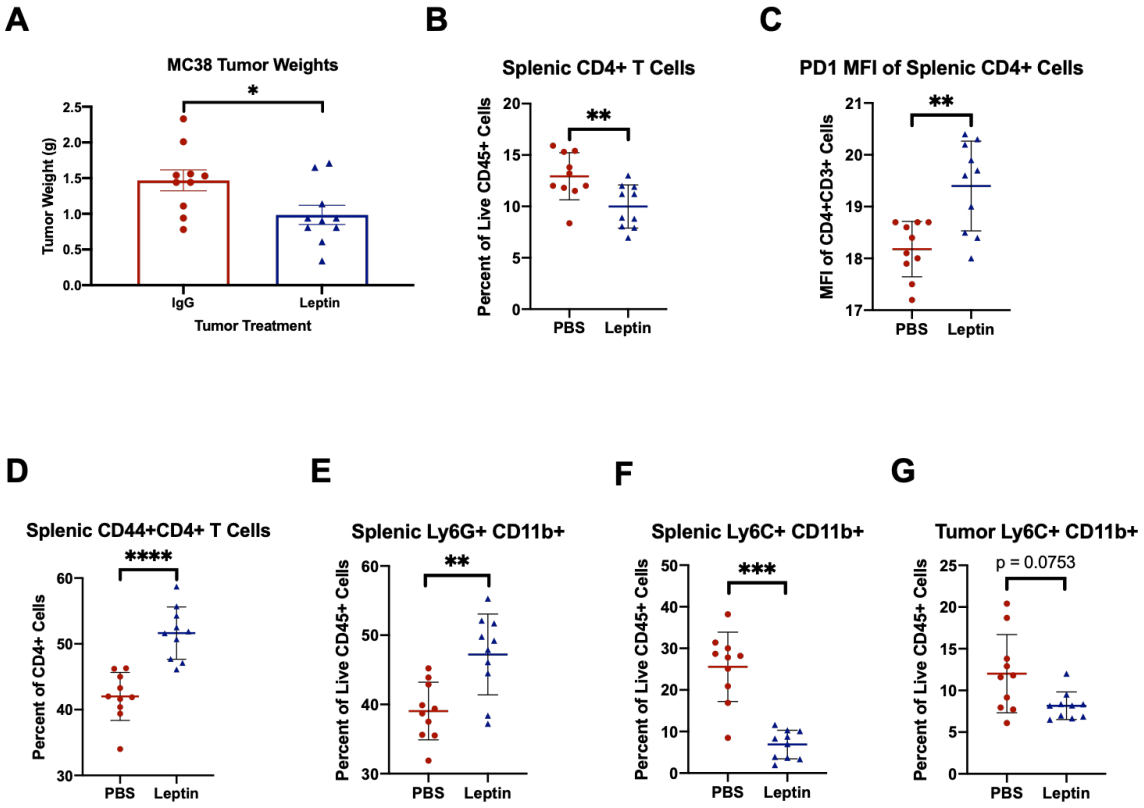


Supplemental Figure A.S6 C57BL/6 male mice on a control standard chow diet (n=10) or 60kcal high-fat diet (n=10) for 12 weeks were injected with 2.5 x 10^5 MC38-CEA1 cells in the right flank. On day 5 post tumor-injection, mice were injected with either 200 µg IgG control antibody or anti-PD-1 antibody, and the injections continued every two days until tumors were collected on day 16 post-injection. (A,B) Spleens from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry. Frequency from low-fat diet IgG treated (LFD IgG, n=10), low-fat diet anti-PD-1 antibody treated (LFD PD1, n=10), high-fat diet IgG treated (HFD IgG, n=10), and high-fat diet anti-PD-1 treated (HFD PD1, n=10) mouse spleens. (A) Frequency of CD44$^+$ splenic T cells. (B) PD1 expression by MFI on splenic CD4$^+$ T cells. Data in this figure are all depicted as mean± S.E.M., with all individual points shown. Ordinary one-way anova test *p* values shown. **C.** Frequency of IFNγ+ cells in the CD8+ TIL population. Data in this figure are all depicted as mean± S.E.M., with all individual points shown. Ordinary one-way anova test *p* values shown *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

APPENDIX B

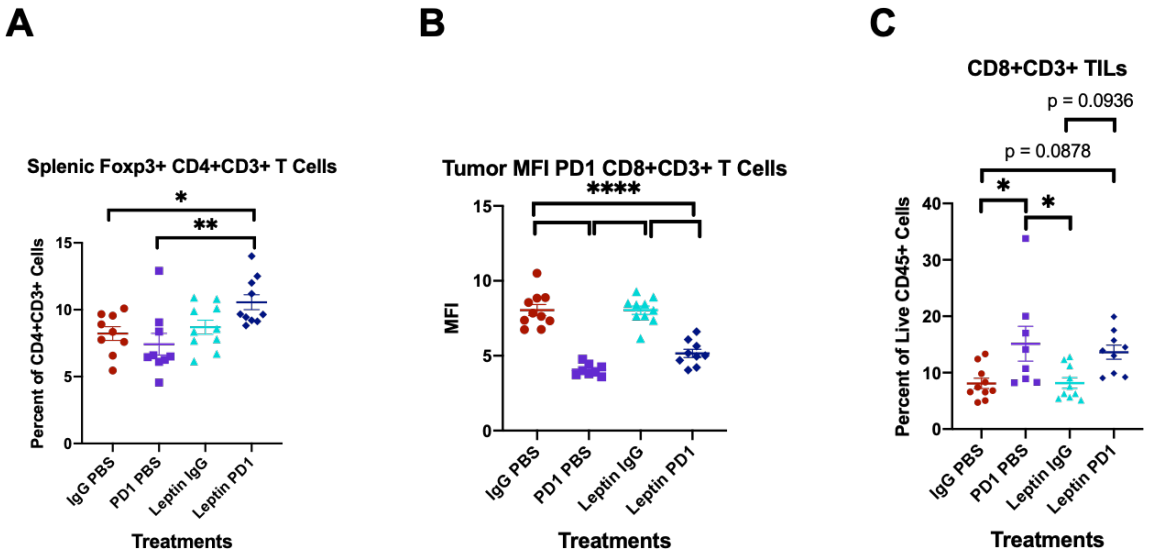
SUPPLEMENTARY MATERIAL FOR CHAPTER III

Leptin Induces a Decrease in Splenic CD4$^+$ T Cell Frequency and Increases CD4$^+$ T Cell Exhaustion Profile



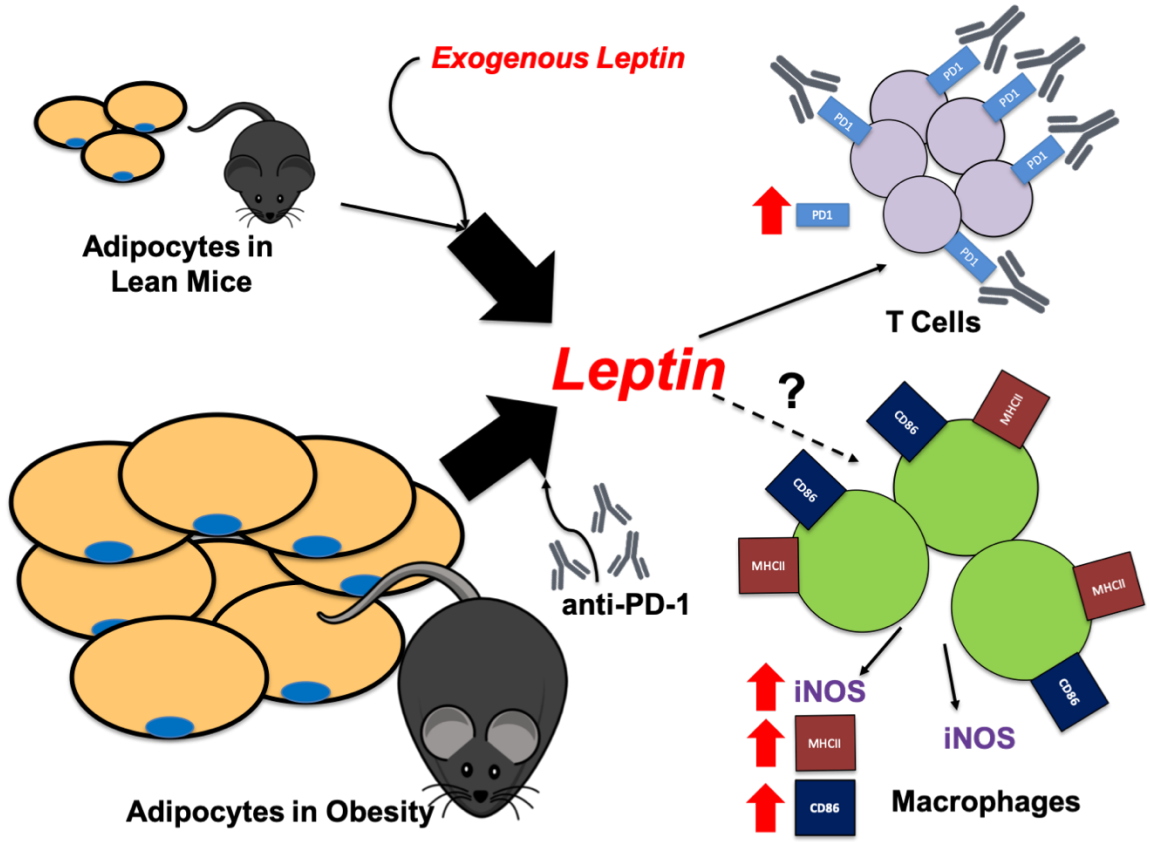
Supplemental Figure B.S1 Five-week old C57BL/6 male mice on a control standard chow diet were injected with either leptin (1 µg/g body weight) or PBS control twice a day for two weeks before subcutaneous injections with 10^5 MC38-CEA1 cells were given in the right flank. Leptin injections continued throughout tumor growth. Spleens and tumors from mice with MC38-CEA1 tumors were collected 16 days post-injection and were processed single cell suspensions, before analyzing by flow cytometry. (A) Tumor weights after 16 days of growth. (B) Frequency of CD4$^+$ splenic T cells. (C) PD1 expression by MFI on splenic CD4$^+$ T cells. (D) Frequency of CD44$^+$ splenic T cells. (E) Frequency of splenic Ly6G$^+$CD11b$^+$ PMN-MDSCs. (F) Frequency of splenic Ly6C$^+$CD11b$^+$ PMN-MDSCs. (G) Frequency of intratumoral Ly6C$^+$CD11b$^+$ PMN-MDSCs Two-tailed Mann Whitney test p values shown. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.

Leptin and ICB Treatment Effects on T Cells



Supplemental Figure B.S2 Five-week old C57BL/6 male mice were given subcutaneous injections with 2.5 x 10^5 MC38-CEA1 cells in the right flank. On day 5 post tumor-injection, mice were injected with either 200 µg IgG control antibody or αPD-1 antibody, and the injections continued every two days. Additionally, on day 5 post-tumor injection, mice received either leptin (1 µg/g body weight) or PBS control twice a day. Treatments for the PBS IgG antibody (n=10), Leptin IgG antibody (n=10), PBS αPD-1 antibody (n=10), and Leptin αPD-1 antibody continued until 16 days post-injection. (A) Frequency of splenic Foxp3$^+$ CD4$^+$ CD3$^+$ regulatory T cells. (B) MFI expression of PD1 in CD8$^+$ CD3$^+$ TILs. (C) Frequency of CD8$^+$ CD3$^+$ TILs. Data in this figure are all depicted as mean± S.E.M., with all individual points shown. Ordinary one-way anova test *p* values shown *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

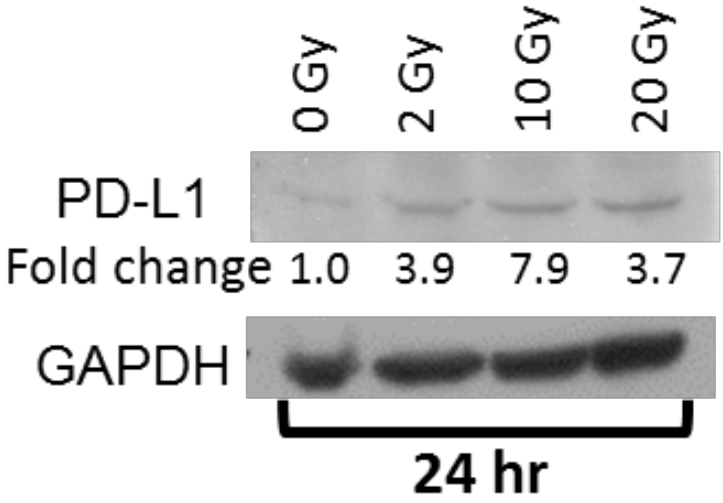
Model for Leptin Repolarization of TAMs in Obese Immunotherapy Experiments



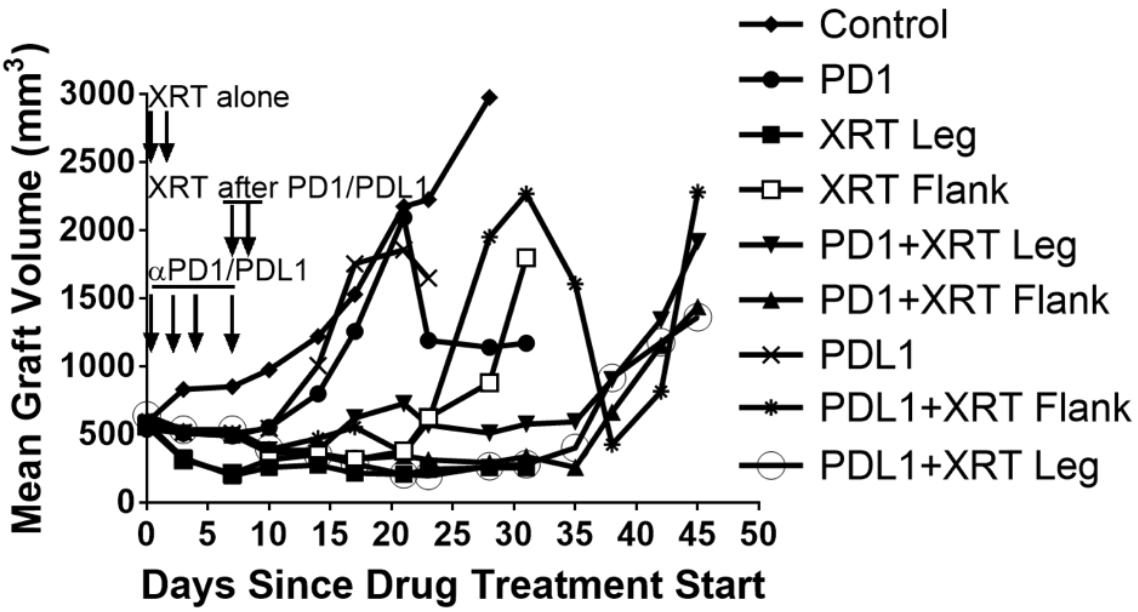
Supplemental Figure B.S3 The experiments support that exogenous leptin in lean mice increases PD1 expression on T cells and increases M1 polarization of macrophages. When treating tumor-bearing obese mice with anti-PD-1 antibodies, we propose that the elevated leptin in conjunction with immune checkpoint blockade are capable of repolarizing tumor-associated macrophages to an M1 phenotype as this repolarization was not observed in lean mice treated with immune checkpoint blockade.

APPENDIX C

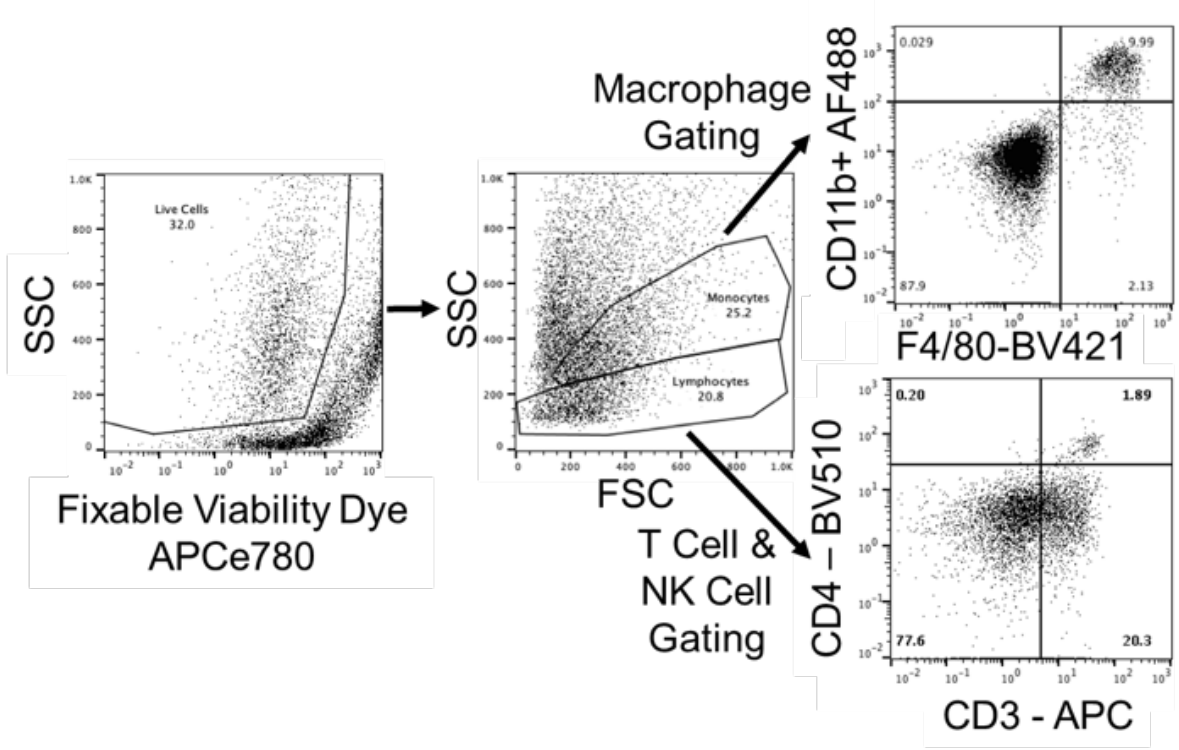
SUPPLEMENTARY MATERIAL FOR CHAPTER IV



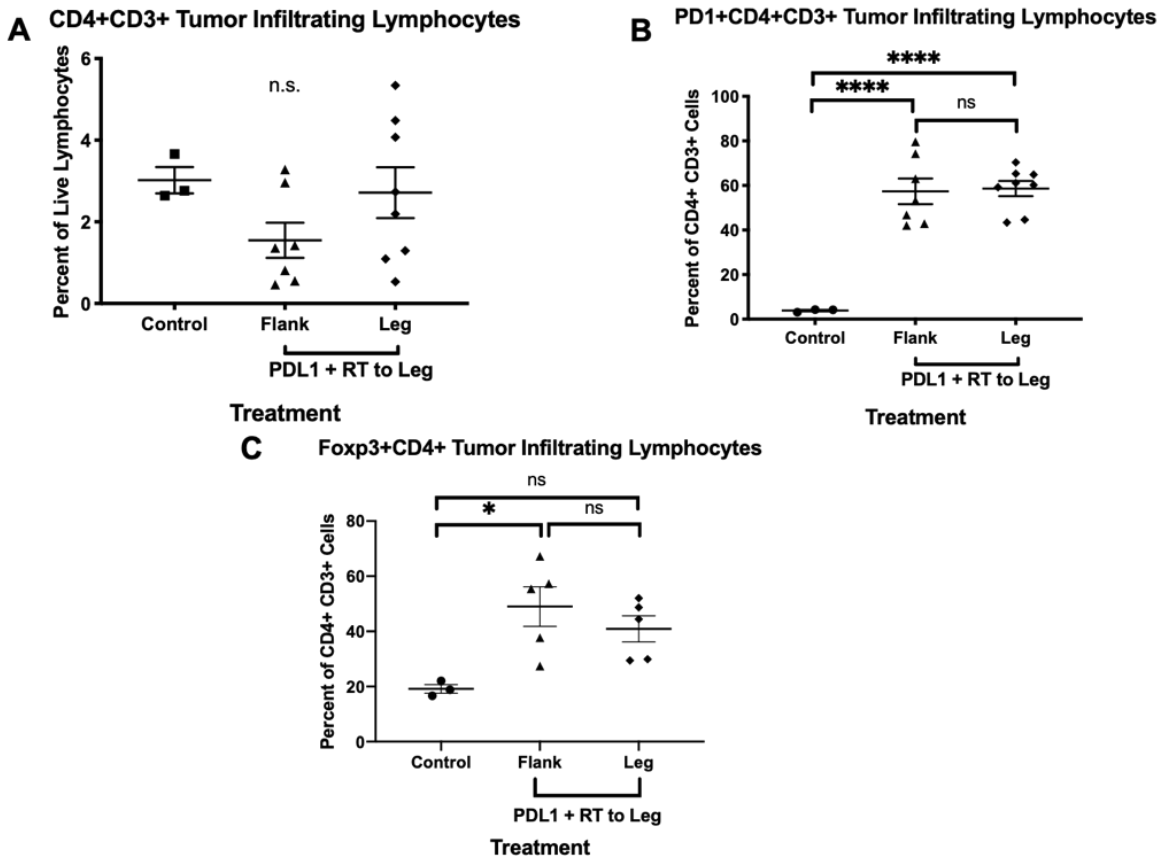
Supplemental Figure C.S1: Myc-CaP cells were treated with radiation at indicated doses and then incubated for 24 hours. Total protein was extracted and probed as indicated. Bands were quantified, corrected for GAPDH expression (ImageJ). Fold change is normalized to 0 Gy (unirradiated) control.



Supplemental Figure C.S2: Mice were treated with the indicated therapy and graft volumes were measured 2-3x per week until a predetermined endpoint was reached. Mean growth curves appear more erratic beyond 3 weeks of treatment due to fewer remaining mice in each group. Error bars not shown for figure clarity.



Supplemental Figure C.S3: Flow cytometry gating strategy. Two panels of fluorophores were used to analyze T cell populations and macrophage/NK cell populations. Only live cells were analyzed by the initial gate using the fixable viability dye APCe780. Next, SSC/FSC measures allowed separate gating of the lymphocyte and monocyte populations, with the fluorophore panels testing each staining characteristic.



Supplemental Figure C.S4: Flow cytometry for CD4+ tumor infiltrating lymphocytes. A and B. Flow cytometry of CD4+ cells within Myc-CaP grafts in mice with untreated control tumors (N=3), flank tumors from mice treated with anti-PD-L1 and XRT to leg tumor (N=7), or leg tumor that received direct XRT and ant-PD-L1 treatment (N=8). Enrichment in the PD1+ CD4+ tumor infiltrating lymphocytes was observed after treatment with anti-PD-L1 antibody in both the irradiated leg tumor and unirradiated flank tumor in the same animals. Additionally, the T regulatory Foxp3+ CD4 T cells population was enriched in the flank tumors. Error bars represent ± SEM; ****$P < 0.0001$, two-way ANOVA test.

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