The Dissertation Committee for Gloria Cecilia Galván Certifies that this is the approved version of the following dissertation:

Obesity and Prostate Cancer Progression: Determining the Role of Macrophages in the Tumor Microenvironment

Committee:

Linda A. deGraffenried, Supervisor

Murali Beeram

Christopher A. Jolly

A. Pratap Kumar

Bob G. Sanders

Obesity and Prostate Cancer Progression: Determining the Role of Macrophages in the Tumor Microenvironment

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Gloria Cecilia Galván

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Obesity and Prostate Cancer Progression: Determining the Role of Macrophages in the Tumor Microenvironment

Gloria Cecilia Galván, Ph.D.

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Supervisor: Linda A. deGraffenried

Obesity is associated with a greater risk of prostate cancer mortality. However, the mechanisms connecting obesity to the progression of prostate cancer remain unknown. This study determined the impact of obesity on the prostate tumor microenvironment (TME) by looking at macrophage recruitment and tumor-associated macrophage (TAM) polarization. Results from the study showed that obese mice had higher macrophage and TAM infiltration into the TME compared to normal weight mice in a PTEN knockout mouse model. To answer mechanistic questions, an *in vitro* model in which adipose stromal cells, prostate cancer cells, and macrophages were exposed to sera from obese or non-obese men, or conditioned media generated under obese or non-obese conditions was utilized. Obese conditions increased expression of recruiting and polarizing molecules in adipose and prostate cancer cells, and of TAM markers in macrophages. Since a high concentration of TAMS in tumors has been linked to progression in prostate cancer, the effects of obesity-induced TAMs on prostate cancer cells were observed by looking at characteristics of an invasive phenotype *in vitro*. Furthermore, the potential of targeting

TAMs with rapamycin was studied, given that mTOR has been shown to be important for macrophage polarization and stabilization. Rapamycin selectively decreased viability of obesity-stimulated TAM-like macrophages compared to M1 macrophages in this model. Data suggest that obesity promotes macrophage infiltration into the prostate tumor microenvironment, and induces TAM polarization through the COX-2/PGE2 pathway. Additionally, the mTOR pathway appears to be involved in the survival of TAMs. This study offers a novel mechanistic approach to treat obese patients with prostate cancer.

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Chapter 1: Introduction

1.1 Prostate Cancer

Prostate cancer (PCa) accounts for 19% of new cancer cases and as many as 1 in 8 men will be diagnosed in their lifetime. Even more importantly, PCa is the second most common cause of cancer deaths in American men (Siegel, 2017). With an estimated 1.1 million cases worldwide in 2012, there is an immense need to identify an effective, nontoxic therapy for PCa patients (Torre, 2012). Currently, treatment plans for PCa patients vary according to disease stage. Early detection accompanied by slow growth typically results in active surveillance, whereas later detection often warrants treatment. Localized treatment options consist of surgery, namely prostatectomy, and radiation therapy, both of which are known to have immediate side effects and can result in permanent erectile dysfunction and incontinence. Systemic treatment options consist of androgen deprivation therapy (ADT) and chemotherapy, which can result in an array of undesirable symptoms in patients. These treatments can be performed alone or in combination depending on the case. However, current estimates suggest that up to half of all PCa patients are over-treated, which is a major concern because overtreatment can result in unnecessary side effects (Loeb, 2014). In more recent years, immunotherapy has shown promise as a less-toxic, more-effective treatment option for cancer patients. Additionally, there is a need to identify biomarkers that distinguish indolent and aggressive forms of PCa, to avoid overtreatment for patients with low risk of progression (Evans, 2009) (Spahn, 2015).

Androgen deprivation therapy reduces circulating levels of testosterone, which is required for disease progression in hormone-dependent PCa, and is considered the standard of care for treating PCa, especially at early stages (Mukherji, 2014). The majority of PCa patients are initially sensitive to ADT, however in advanced PCa responses are generally short-lived and disease progression is very likely (Suárez, 2014). In cases such as these, castration-resistant prostate cancer (CRPC) occurs when the disease progresses despite minimal levels of testosterone, often metastasizing to the bone, and has historically been considered chemo-resistant (Allot, 2013). To date, the mechanisms that drive PCa progression remain unknown.

Inflammation is a well-known hallmark of cancer and has been found to play an important role in tumorigenesis through modulation of immune cells and cytokine signaling, among other mechanisms (Grivennikov, 2010). Chronic inflammation, has been found to affect not only PCa epithelial cells, but the tumor microenvironment (TME) as well (Zeigler-Johnson, 2016) (Chiarugi, 2014). The TME harbors more than cancer cells and includes surrounding fibroblasts, blood vessels, as well as immune, stromal, adipocyte, and endothelial cells, and of particular interest – tumor-associated macrophages (TAMs). Possible sources of inflammation include obesity and diet, which are linked to a more aggressive disease and poorer patient outcomes (Allot, 2015) (Moller, 2015).

While much research has traditionally focused on targeting cancer cells, targeting key players of the TME has become an area of increasing interest in recent years. TAMs provide a viable target for improved outcomes in PCa because of their essential role in the

prostate TME. Moreover, TAMs have been found to act on the TME through a variety of mechanisms, and are associated with higher-grade PCa and metastasis. Since TAMs are known to be pro-tumorigenic, novel therapeutics that target the negative effects of TAMs on PCa could be a viable target for improving outcomes in patients.

1.2 Current Knowledge of Tumor-Associated Macrophages in Prostate Cancer

1.2.1 From macrophages to TAMs

Macrophages originate from the innate immune system and are derived from hematopoietic stem cells (HSCs) in the bone marrow (Cortez-Retamozo, 2012). HSCs continuously proliferate and shed their progeny into the bloodstream as promonocytes, which develop into monocytes and extravasate into tissues exhibiting low-grade inflammation, such as neoplastic tissue (Lewis, 2006) (Mallat, 2014). Once in the tissue, promonocytes undergo differentiation into macrophages of varying phenotypes (Cortez-Retamozo, 2012). While the mechanisms by which they differentiate have not been fully elucidated, it is known that macrophages undergo polarization into many different subtypes. Of these subtypes, the two most commonly studied lie on opposite ends of the polarization spectrum: M1 and M2 macrophages (Lanciotti, 2014).

M1, or classically activated, macrophages predominantly display cytotoxic and pro-inflammatory effects in the TME, while M2 macrophages are anti-inflammatory and tumor promoting. M1 macrophages are characterized by high levels of IL-12 and IL-8/CCL

production and cell surface expression of CD80 and CD86. M2 macrophages are characterized by the excretion of growth factors TGF-β, VEGF, and cell surface expression of CD163 and CD206 (Mills, 2015). In the TME, pro-tumorigenic TAMs display the same cytokine profiles as the alternatively polarized M2 macrophages (Rhee, 2016) (Liu, 2015). Macrophage polarization is dictated by environmental stimuli, and it is known to be a reversible process.

1.2.2 Mechanisms by which TAMs modulate the tumor microenvironment

Following macrophage recruitment and polarization to the M2 phenotype, TAMs can modulate the microenvironment and lead to cancer progression. TAMs are known to promote an immunosuppressive microenvironment by producing anti-inflammatory molecules, and to drive tumor progression by enhancing tumor angiogenesis, survival, and metastasis (Soave, 2016) (Hanahan, 2012). In the TME, TAMs tend to accumulate in poorly vascularized and necrotic areas (Coffelt, 2009). Angiogenesis is a known hallmark of cancer which contributes to worse outcomes by orchestrating the formation of new vessels to the TME thus supporting tumor growth by increasing oxygen and nutrient availability (Hanahan, 2011). Clinical human studies have shown a correlation between high TAM concentration, increased microvessel density, and poor prognosis in a variety of cancers, including prostate (Ohno, 2004) (Li, 2002) (Onita, 2002). TAMs contribute to angiogenesis by secreting a variety of pro-angiogenic mediators, such as basic fibroblast growth factor, thymidine phosphorylase, urokinase-type plasminogen activator (uPA),

adrenomedullin (ADM), VEGF via the NF-κB signaling pathway, IL-6, IL-10, HIF-1, and HIF-2 (Hanahan, 2011) (Chanmee, 2014).

Tumor survival, or the ability to resist apoptotic triggering of cell death, is another hallmark of cancer (Hanahan, 2011). Current literature suggests that TAMs are associated with increased tumoral survival through a variety of mechanisms. Some of the mechanisms include increased production of IL-6 through tyrosine phosphorylation of STAT3, and thereby its target genes TGF-β1 and HIF-1α, which function to promote survival of tumor cells to hypoxia (Jeong, 2017) (De Beule, 2017) (Zhou, 2015). Another mechanism is through the release of IL-1β, which inactivates GSK3β, enhances Wnt signaling, and confers a resistance to TRAIL-induced apoptosis (Kaler, 2010).

Interestingly, TAM production of IL-6 and the resultant STAT3 signaling has also been implicated in promoting the expansion of CD44 cancer stem cells in the TME and epithelial to mesenchymal transition (EMT) through the TGF-β1 signaling pathway (Wan, 2014) (Fan, 2014). EMT progression of cancer cells to a more stem-like phenotype is known to confer worse cancer outcomes via invasive and metastatic mechanisms, as epithelial cancer cells gain invasive and migratory capacities while losing cell to cell contact (Singh, 2010).

TAMs stimulate metastasis by promoting cancer cell invasion through mechanisms such as tissue remodeling and altering the extracellular matrix (ECM) structure. Some *in vitro* studies have demonstrated that TAM involvement in cell invasion is related to their release of metalloproteinases (MMPs), such as MMP-9 and MMP-2, and by secreting

epidermal growth factor (EGF) (Kessenbrock, 2010) (Goswami, 2005). TAMs are the primary source of EGF, which is one of the main drivers of EMT, and is associated with metastasis and invasive tumors. Another less studied mechanism is a TAM-dependent alteration of the collagen structure, which allows for increases motility properties in cancer cells (Levental, 2009). In PCa *in vivo* models, macrophage depletion therapy and anti-CCL2 antibodies have been shown to reduce the macrophage infiltration in tumors and decrease the incidence of bone and lymph node metastasis (Kim, 2012) (Loberg, 2012). Furthermore, accelerated metastasis has been observed in tumors that overexpress the macrophage recruiter CSF-1 (Lin, 2001).

In summary, TAMs are known to promote cancer aggressiveness by driving angiogenesis, accelerating tumor growth and survival, and stimulating metastasis.

1.2.3. TAMs and prostate tumor grade

Currently, PCa is evaluated at the time of diagnosis using the Gleason scoring system and a prostate specific antigen test (PSA) (Kattan, 2007). Gleason scores indicate the severity of a patient's PCa, ranging from one to nine, and are typically grouped into three categories – low, medium, and high – grades. Gleason scores are derived from prostate biopsies in which each section taken from the patient's prostate is assigned a number based on the severity of their cancer in that section, the numbers are then summed to form an overall score (Epstein, 2010).

A new scoring system for PCa, called the 5 Grade Group system has emerged. While this system promises more accurate stratification of PCa grades and simplification

of scoring to avoid patient confusion, Gleason scores are still often used in clinical settings. Most practicing physicians are accustomed to using Gleason scores, and may or may not be trained to use the new scoring system. In addition, when the 5 Grade Group scoring system was introduced, it was recommended that patients be given both their Gleason score and their 5 Grade Group score to avoid confusion (Chen, 2016). This approach has allowed Gleason scores to persist clinically, and, oftentimes, solely.

The other common method for evaluation of PCa used in clinical settings is the prostate-specific antigen test (PSA test), which is used to determine the possible presence of PCa in an individual. The primary difference between the PSA test and Gleason score is that the PSA test is minimally invasive. PSA levels are obtained by performing a routine blood test, whereas Gleason scores are assigned after a patient has undergone a biopsy. Biopsies usually occur after an elevated PSA level, which is considered to be above 4.0 ng/mL. The PSA test measures prostate-specific androgen levels, a protein produced by cells of the prostate gland. Unlike the Gleason score, a patient's PSA level can be elevated for non-cancerous reasons, such as benign prostatic hyperplasia (BPH), urinary tract infections, sexual intercourse, medical procedures, rectal exams, and age. As such, men with an elevated PSA usually undergo a biopsy to confirm the presence and evaluate the grade of a cancerous lesion.

While low-grade Gleason scores (1-3) warrant active surveillance consisting of bi-annual PSA tests and high-grade scores (7-9) typically require prostatectomy, the treatment route for medium-grade scores (4-6) is dictated by the interpretation and choice of

individual clinicians and their patients. Decisions are made on a case-by-case basis because there is no reliable biomarker to determine whether a PCa tumor will progress to high-grade or remain medium-grade. Thus, medium-grade PCa is often over-treated to prevent potential progression. This is problematic because aggressive treatment of non-threatening, medium-grade prostate cancers may be more detrimental to the patient's health than living with cancer. There is a substantial need to find a reliable prognostic biomarker to reduce the overtreatment of PCa, especially for patients with mid-grade tumors.

One potential biomarker for PCa progression is TAM concentration. A 2011 study published in the International Journal of Cancer found that TAMs promoted carcinogenic conditions in breast tissue (Jézéquel, 2011). Other studies have linked infiltration of TAMs to cancer progression, but few studies have evaluated TAM concentration in PCa (Shimura, 2000). In fact, studies of TAM concentration within PCa have analyzed TAM concentration following hormonal therapy, but not as a biomarker to indicate cancer progression (Nonomura, 2010). Accordingly, TAM concentration represents a promising potential biomarker for PCa progression, and further research is needed in this area.

1.2.4 Current clinical thoughts to target tumor-associated macrophages

Targeting the TME has been shown to be an effective strategy for treating cancer, given that noncancerous cells can enable multiple mechanisms that enhance tumor progression (Hanahan, 2012). As demonstrated in this review, it has been established that TAMs play an important role in solid tumors. Therefore, suppression of the pro-tumorigenic activities of these cells in the TME can be used as a therapeutic strategy in

conjunction with current traditional and new therapies, such as immunotherapies, to improve response to treatment, minimize the need for surgery, or prevent recurrence. In the case of macrophages in the TME, targeting of cytokines and chemokines that drive macrophage polarization into a tumor-associated phenotype could prevent the polarization of macrophages into TAMs. Most studies to date have aimed to inhibit M2 polarization, but few have considered the reprogramming of already polarized M2 macrophages back to the M1 phenotype, which could be a promising way to intervene given that M1 macrophages are anti-tumorigenic (Heusinkveld, 2011). However, macrophage reprogramming is a topic under current investigation.

Current therapeutic interventions targeting TAMs aim to block macrophage migration to the tumor and inhibit specific TAM functions, including the production of immunosuppressive and angiogenic factors. Inhibitors of CCL2 and CXCL12, the main chemokines that direct macrophage recruitment into tumors have been implicated in blocking TAM recruitment, thus blunting their influence on the TME. In PCa specifically, one study demonstrated that CCL2 neutralizing antibodies decreased the growth of prostate cancer cells and inhibited metastasis by blocking macrophage infiltration to the tumor site (Mizutani, 2009). Other therapeutic strategies include CSF1 inhibitors, which block the CSF1-CSF1R interaction and inhibit macrophage migration, proliferation, and survival. In PCa, compared to ADT alone, a combination treatment including CSF1R inhibitors and androgen blockade resulted in decreased TAM recruitment, repressed tumorigenesis, and delayed tumor regrowth in mice (Escamilla, 2016).

Several studies have aimed to target macrophages in different ways. One way is by targeting the expression of membrane proteins, such as Toll-like receptors (TLRs), which are expressed on the membrane of macrophages. TLR agonists are being investigated in the reprogramming of M2 to M1 macrophages through receptor-mediated activation signals. It's worth noting here that certain cytokines which guide macrophage polarization need to be present as well, thus T-cell responses must be modulated in order to develop an effective repolarization strategy. Among other therapies that inhibit TAM function, Tasquinimod targets pro-metastatic signaling from TAMs (Olsson, 2015). Other therapies include angiogenic inhibitors that target pro-angiogenic proteins, such as VEGF. However, these therapies have been shown to only be effective short-term due to tumor resistance (Casanovas, 2005). Additionally, clodronate liposomes, a macrophage-depletion therapy, have been shown to result in reduced tumor growth and angiogenesis in mice by targeting monocytes and circulating macrophages (Zeisberger, 2006). Although, they have not yet been widely studied in this setting, non-steroidal anti-inflammatory drugs (NSAIDs) have also been proposed as a potential therapy to target macrophage-related activities, given that they can inhibit prostaglandins involved in macrophage polarization by targeting the cyclooxygenase-2 (COX-2) pathway (Pollard, 2004).

Several studies have already shown that TAM-targeted therapies can involve limiting the function of TAMs, preventing the polarization of macrophages into a pro-tumorigenic phenotype, blocking TAM infiltration into tumors, or inducing the reprogramming of TAMs (Ruffell, 2012). One of the benefits of macrophage-targeted

therapy is that macrophages are not likely to develop drug resistance because they do not possess malignant mutations due to their stable genome (Condeelis, 2006). In addition to targeting tumor-associated macrophages directly, the presence of other immune cells that interact with TAMs in the TME should be evaluated as potential immunotherapeutic targets. In this case, combination therapies could be used to create a synergistic and more efficacious treatment.

1.2.5 Summary

With PCa being the second most common cause of cancer deaths in American men and the baby boomer population reaching the age where PCa is most common, there is a significant need for better treatments and increased understanding of PCa. While varying PCa disease stages warrant different treatment routes, elucidating which PCa patients will require more aggressive treatment will aid in improving outcomes. One such method for discovering which cancers need aggressive treatment, is to determine an effective biomarker that can assist in the distinction between indolent and aggressive forms of PCa. TAMs, associated with a more aggressive PCa, serve as a promising potential biomarker for distinguishing which PCa patients need more aggressive treatment. While not carcinogenic themselves, TAMs modulate the TME and have profound effects on angiogenesis, metastasis, survivability, and the EMT pathway. The importance of TAMs in the prostate TME has been demonstrated by the relationship between TAMs and PCa grade where higher grades exhibit increased concentrations of TAMs. Moreover, TAM-targeting therapies are showing promise in pre-clinical and clinical studies. Research into

blocking macrophage infiltration, inhibiting chemokines that induce TAM polarization, and repressing angiogenic and metastatic functions of TAMs have all produced positive results. However, further research is still needed to determine if these therapies can be used alone or are more effective in combination with immunotherapy or chemotherapy.

1.3 Obesity and Prostate Cancer

1.3.1 Obesity and prostate cancer progression

According to the National Cancer Institute, the 5-year survival rate for localized prostate cancer decreases from 99% to 30% once it has metastasized. Obese men are more likely to experience invasive and metastatic prostate cancer in comparison to their normal weight counterparts, accompanied with an elevated risk of death due to the disease (Freedland, 2005) . Although some studies have attributed the connection between obesity and aggressive disease to a harder detection procedure in obese men, this does not fully explain the connection between the two. Moreover, there is a study showing a positive association between obesity and high-grade prostate cancer in men who all underwent prostate biopsies (Gong, 2006). Another study showed that being overweight or obese increased the risk of dying from prostate cancer specifically (Vidal, 2017). This would suggest that obesity could be changing the biology of tumors promoting a more aggressive phenotype. Some of the biological mechanisms involved in this connection might be the alteration of hormone levels and metabolic changes, given that obese men usually have

higher levels of insulin and leptin. Also, it has been suggested that peri-prostatic adipose may have a role in prostate cancer aggressiveness, as it correlates with higher Gleason scores (Finley, 2009), possibly by driving the recruitment of inflammatory and immune cells to the tumor microenvironment. The presence of these cells should continue to be studies, as immune cells sometimes comprise more than 50% of tumors (Mills, 2016). Macrophage recruitment in obesity-driven breast cancer development has been related to the overexpression of macrophage recruiting proteins, such as MCP-1, in human adipose stromal cells (Arendt, 2013).

There is enough evidence connecting obesity and advanced-prostate cancer to establish obesity as a risk factor for aggressive disease in prostate cancer and prostate cancer mortality. Given the prevalence of obesity in the United States and its growth in developed countries, there is a need for a greater understanding of the mechanisms mediating obesity-induced prostate cancer progression.

1.3.2 Obesity and macrophage polarization

Olefsky et al. have shown that M1 macrophages dominate in fat tissue due to the pro-inflammatory state involved with obesity (Olefsky, 2010). However, in the context of cancer, differentiation of macrophages may change. The state of obesity is not only associated with the increased infiltration of macrophages and adipocytes into the TME, but also with the induction of reciprocal signals between the inflammatory and tumor cells to promote an M2 or TAM phenotype in macrophages. A few studies have shown an interesting association between PGE2 production by cancer cells and the ability of

macrophages to produce IL-10, suggesting that PGE2 could be involved in macrophage polarization (Heusinkveld M, 2011) (Nakanishi Y, 2011). IL-10 has been noted to inhibit immune response against tumor cells by inhibiting the production of inflammatory cytokines (Schmieder, 2012). PGE2 can be secreted by different cell types including monocytes, macrophages, and cancer cells acting in an autocrine and paracrine manner. The role of adipocyte-related inflammation in guiding macrophage polarization in the tumor microenvironment remains poorly understood. Therefore, the mechanisms by which adipocytes, macrophages, prostate epithelial cells and the obesity- related programming in the TME are causing prostate cancer progression, should be elucidated.

1.4 DISSERTATION OBJECTIVES

Obesity is associated with an exponential increase of prostate cancer mortality (Allot EH, 2013). While some studies have focused on the hormonal component of the disease, this study suggests that obesity-associated inflammation in the tumor microenvironment is also responsible for the link between obesity and prostate cancer progression. The objective of these studies was to determine how obesity may drive changes in the tumor microenvironment, which in turn could contribute to prostate cancer progression. The focus of this study is on the presence and the role of macrophages in the tumor microenvironment, while hypothesizing that obesity drives prostate cancer progression by inducing macrophage infiltration into the TME, and by programming macrophages towards TAMs. The rationale behind this hypothesis was based on preliminary data showing that obesity induces monocyte migration towards cancer cells

and increases levels of circulating monocyte chemotactic protein-1 (MCP-1/CCL2). CCL2 directs recruitment of macrophages during inflammation and has been found to be elevated in prostate cancer (Lu Y, 2006). These data suggest that, within the context of obesity, MCP-1 could be enhancing macrophage recruitment resulting in increased inflammation. In addition, it has been observed that prostaglandin E2 (PGE2) molecule, whose production has been associated with obesity, appears to be involved in macrophage polarization. Consequently, it is thought that PGE2 may play a mechanistic role in obesity-induced TAM polarization.

In this study, the composition of the tumor microenvironment in an obese state was determined in an *ex vivo* model. Furthermore, the role of obesity in macrophage polarization towards tumor-associated macrophages, as well as the mechanism of action were explored *in vitro*. Lastly, the effect of obesity-induced tumor-associated macrophages on prostate cancer progression, and potential ways to target TAMs were investigated.

1.4.1 Research significance

Understanding how epithelial cells, macrophages and adipocytes interact with each other in the prostate microenvironment in the context of obesity will provide new knowledge about prostate cancer progression in obese men. Identifying macrophage subtypes in the microenvironment of a prostate tumor is an important stepping stone in the development of novel treatments for obese patients with prostate cancer. In addition, the identification of progression mediators can serve to determine potential therapeutic targets.

Chapter 2: Obesity regulates the macrophage population in the prostate tumor microenvironment

2.1 INTRODUCTION

Obesity affects more than one third of U.S. adults. According to data from the Center for Disease Control and Prevention (CDC), 34.9%, or 78.6 million, adults are obese in the United States (Ogden, 2012). The obese state has been associated with chronic inflammation due to the infiltration of immune cells to the adipose tissue resulting in an increased risk of developing or driving the progression of certain cancers (Iyengar, 2015) (Hursting, 2012).

Sixty-five percent of men aged 60-70 have prostate cancer, and although mortality risk [1]from prostate cancer is low, it is significantly impacted by obesity (Calle, 2003).A study done with the Health Professionals Follow-up Study cohort, which is comprised of 51,529 male health professionals, found a significant association between high BMI and high intake of total energy with risk of fatal prostate cancer (Giovannucci, 2007). Compared to lean men, obese men have higher resistance to treatment, higher-grade prostate cancer, and an increased incidence of metastasis (Freedland, 2005). This would suggest that obesity is a significant contributing factor to the aggressiveness and

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invasiveness of prostate cancer, although the mechanisms that connect obesity and prostate cancer progression have not been defined. Previous studies have suggested that peri-prostatic adipose tissue may play a role in prostate cancer aggressiveness (Finley, 2009). Nevertheless, the role of the tumor microenvironment in the progression of this disease remains to be elucidated.

The complex tissues that comprise solid tumors are more than just cancer epithelial cells. Fibroblasts, adipose cells and tumor-associated macrophages (TAM) are some of the main non-cancer cells present in the tumor microenvironment of solid tumors (Hanahan, 2012). It is important to note that in an obese state, with the presence of increased fat tissue, the recruitment of these cell types, especially macrophages, may be induced. Macrophages originate as blood monocytes and get recruited by tumor-derived chemokines, such as CCL2. CCL2 (also known as monocyte chemoattractant molecule, or MCP-1) is one of the main modulators of macrophage recruitment, and has been significantly correlated with macrophage accumulation in tumors and associated with cytokines typically secreted by TAMs (Fujimoto, 2009) (Ueno, 2000). Furthermore, it has been demonstrated that CCL2 expression is increased by obesity-related effects, such as elevated circulating levels of insulin and higher adipocyte concentration (Arendt, 2013).

Macrophages are plastic cells, which can be differentiated into certain phenotypes depending on the signals present within their particular microenvironment (Ruffell et al. 2012). This differentiation process is called polarization, and it produces, generally, macrophage populations which are classified into M1 and M2 subtypes. It has been widely

established that M1 macrophages are also known as classically activated and display pro-inflammatory functions, whereas M2 macrophages are alternatively activated and are mainly involved in anti-inflammatory processes. M2 macrophages contribute to immunosuppression and pro-tumorigenic activities by producing anti-inflammatory molecules such as interleukin-10 (IL-10), transforming growth factor beta (TGF-beta), and arginase-1. Similarly, they produce vascular endothelial growth factor (VEGF-A) and matrix metalloproteinase-9 (MMP-9), which are associated with metastasis (Mantovani et al. 2002). However, not all macrophage populations neatly fit into the M1/M2 classification and, consequently, more subtypes have emerged in recent years. In addition to these phenotypes, a TAM subtype has been classified as M2-like and has been previously correlated with tumor progression, metastasis and a poor prognosis for different types of cancer, including prostate cancer (Sica, 2008) (Lewis, 2006). The mechanism by which macrophages are polarized into tumor-associated macrophages remains to be elucidated, although, previous studies have shown an association between PGE2 production by cancer cells, and the ability of macrophages to produce M2-related cytokines, suggesting that PGE2 could be involved in M2/TAM polarization (Heusinkveld, 2011) (Nakanishi, 2011). Furthermore, PGE2 and cyclooxygenase-2 (COX-2), the enzyme responsible for PGE2 production, have been shown to be elevated in obesity (Bowers, 2015). Therefore, it was thought that this pathway could be related to obesity-induced TAM polarization.

In this study, it was aimed to determine how obesity impacts the tumor microenvironment, specifically the macrophage population with a novel *in vitro* and *ex vivo* model of prostate cancer and obesity.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines and reagents

Androgen-sensitive prostate cancer cell lines, LNCaP and, LAPC-4, were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). LNCaP and LAPC-4 cells were utilized due to their similarities in the expression of the androgen receptor and androgen sensitivity. U937 monocytes (ATCC) were polarized to macrophages at the time of seeding for experiments by adding 10.0 ng/mL phorbol 12-myristate 13-acetate (TPA) (Sigma) and incubating for 48 hours, as previously described (Bowers, 2014). U937 cells were used because of their monocytic characteristics which gives them the ability to be differentiated into other types of cells. Prostate cancer and monocytic cell lines were grown in RPMI-1640 (GIBCO Life Technologies) containing penicillin and streptomycin plus 10% fetal bovine serum (FBS). Pre-adipocytes were a generous gift from Dr. Rong Li from UTHSCSA, where they were isolated from patients undergoing elective surgery. In culture, they were maintained in DMEM/F12 1:1 (GIBCO Life Technologies) supplemented with 10% FBS. All cell lines were maintained in a 5% (v/v) CO_2 humidified incubator at 37°C. Celecoxib was purchased from Sigma.

2.2.2 Serum samples

Sera from male subjects were purchased (Equitech) and pooled by BMI category (Obese: BM I≥ 30 kg/m², Non-Obese: BMI < 25 kg/m²).

2.2.3 Conditioned media

Conditioned media (CM) were generated by seeding $2x10^5$ prostate cancer cells or pre-adipocytes per well in 6-well plates. Cells were serum-starved for 6 hours and then exposed to 2% pooled sera in serum-free media (SFM). After 1 hour of exposure, cells were rinsed with phosphate buffered saline (PBS) and incubated in serum-free media for 24 hours. Conditioned media were collected after 24 hours and stored at -20°C for later use. When assessing the impact of COX-2 inhibition, LNCaP and LAPC-4 were pre-treated with 30μmol/L celecoxib for 1 hour before and during exposure to sera.

2.2.4 Recruitment assay

Matrigel chambers (BD Biosciences) were used to determine macrophage recruitment. Conditioned media from prostate cancer cells were used as a chemoattractant in the lower chamber while macrophages were seeded in the upper chamber. After 24 hours, cells were fixed in 95% ethanol, stained with 1.0% crystal violet, and images were captured.

2.2.5 Quantitative PCR

mRNA levels of CCL2 and COX-2 were measured in pre-adipocytes and prostate cancer cell lines following a 24-hour exposure to 2% sera from obese or non-obese male subjects in SFM. mRNA levels of TGF-beta, IL-10, VEGF-A, arginase-1 and MMP-9 were measured in macrophages after a 24-hour incubation in prostate cancer cell CM. Total RNA extraction was performed with TRizol (Invitrogen). cDNA was synthesized by

(Applied Biosystems) a reverse transcription system per manufacturer's instructions. Quantitative RT-PCR was done utilizing SYBR® Green (Life Technologies) following the manufacturer's recommended cycling conditions. Relative mRNA levels were normalized to Actin mRNA for quantitative evaluation.

2.2.6 Immunohistochemistry

Male C57bl/6 mice were fed an ad libitum high fat (60% kcal fat) or low fat (10% kcal fat) diet for 12 weeks, and induced a prostate-specific PTEN deletion at 6 weeks of age as described in a previous study (Luchman et al. 2008). Histologic analyses included the male reproductive tract of mice. Tissues were fixed in 10% formalin, embedded in paraffin and sectioned transversely. Slides were stained with hematoxylin and eosin (H&E). Prostate lesions were diagnosed from hyperplasia to invasive adenocarcinoma using a histopathological method. To determine macrophage and TAM content in the prostate tissue of these mice, immunohistochemistry (IHC) was conducted on the slides with the use of primary antibodies F4/80 and CD206 (Abcam). Slides were scanned and quantitative analyses were conducted with the use of the software ImageScope (Aperio Technologies).

2.2.7 Prostaglandin E2 concentration

The concentration of PGE2 in conditioned media from prostate cancer cells was measured by enzyme immunoassay using the PGE2 Parameter Assay Kit (R&D Systems) per manufacturer's instructions.

2.2.8 Statistical analyses

Values are presented as mean ± standard deviation. The difference between two different experimental conditions was measured using the Student's *t* test. Significance was set at p value of <0.05.

2.3 RESULTS

2.3.1 Obesity induces macrophage recruitment

Obese conditions increased macrophage recruitment in vitro (**Figure 2.1**) (A). Furthermore, obese mice had higher TAM (CD206+) concentration than non-obese mice when comparing those with high-grade tumors only (B and C), and higher body weight overall (E). When exposed to sera from obese men, CCL2 expression was 75% higher in ASC compared to those treated with sera from non-obese men. Similarly, obese conditions resulted in 58% higher CCL2 expression in LNCaP compared to non-obese conditions. While CCL2 expression increased under obese conditions in LAPC-4 cells, the results were not statistically significant (D and E).

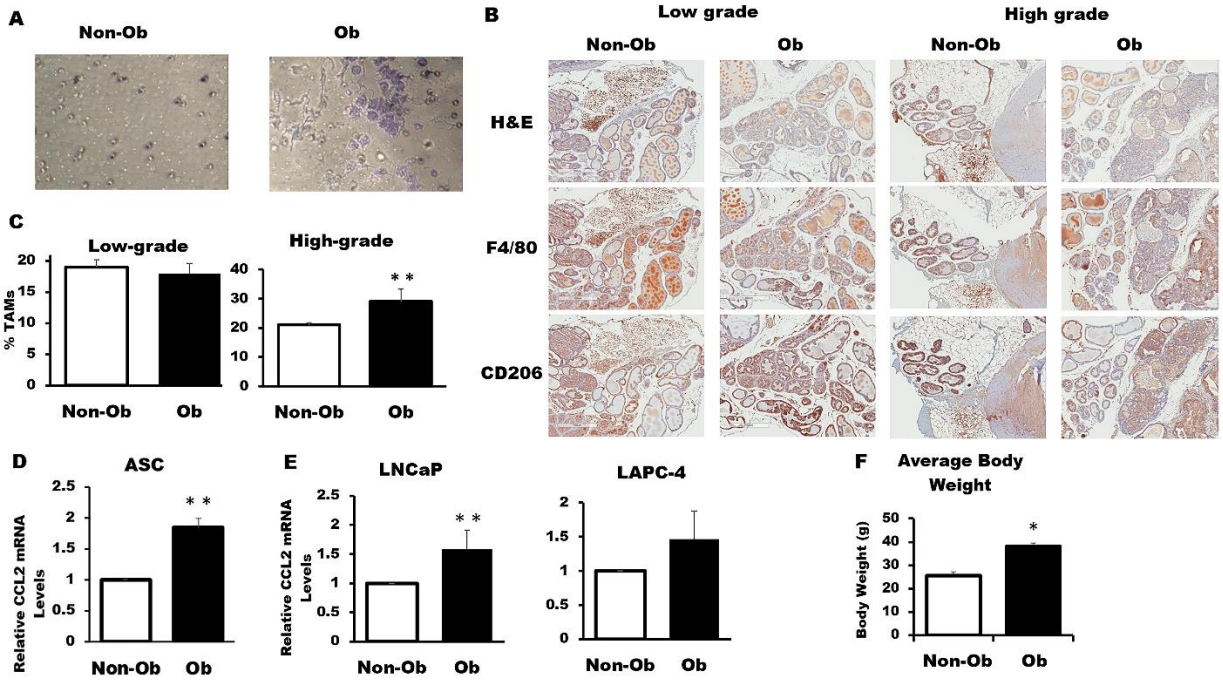

Figure 2.1: Obesity induces macrophage recruitment.

Macrophage recruitment increased under obese compared to non-obese conditions *in vitro* (A). Obesity increased TAM infiltration in mice with high grade-tumors, but not low-grade tumors (B, C). CCL2 expression increased in ASC (D), and in LNCaP and LAPC-4 (E) after exposure to sera from obese or non-obese men. Average body weight was higher in the obese group than in mice under the control diet (E). *, $p<0.05$ and **, $p<0.02$ in comparison to Non-Ob.

2.3.2 Obesity favors polarization of macrophages towards a tumor-associated phenotype through paracrine signaling

Given the finding that obese conditions induce macrophage recruitment, the next step was to determine whether they could cause macrophage polarization towards a specific phenotype. Obesity is usually thought to induce M1 macrophages because of the inflammatory component associated with this state; however, the signals induced by the microenvironment may be altered in the presence of cancer. An *in vitro* model was used in which macrophages were exposed to conditioned media generated from prostate cancer cell lines exposed to pooled sera from obese (Ob) or normal-weight men (Non-Ob). In this model, it was tested if circulating factors in the serum could induce cancer cells to produce polarizing molecules that would induce macrophage differentiation and if there would be a difference between obese and non-obese conditions. Following a 24-hour exposure to Ob conditioned media, TGF-beta, and IL-10 expression in macrophages was higher compared to cells exposed to Non-Ob conditioned media (Figure 2.2). In macrophages treated with conditioned media from LNCaP cells, the Ob condition increased TGF-beta expression threefold and IL-10 expression by 52% in comparison to Non-Ob (A). Macrophages treated with conditioned media from LAPC-4 cells showed increased expression levels of IL-10 and Arginase-1, by 62% and 92%, respectively, in the Ob compared to the Non-Ob condition (B).

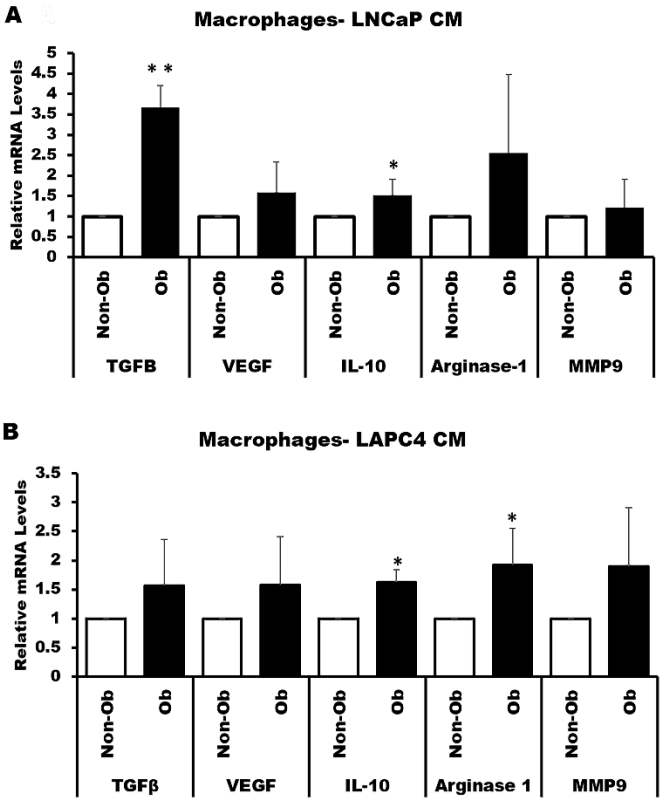

Figure 2.2 Obese-like conditions resulted in higher paracrine induction of TAM markers than non-obese conditions.

To determine if obesity induces a TAM-like phenotype in macrophages, macrophages were treated with conditioned media from prostate cancer cells, LNCaP (A) and LAPC-4 (B), and the expression of TAM markers (IL-10, TGF-beta, VEGF-A, Arginase-1 and MMP-9) was observed by qPCR. *, $p<0.05$ and **, $p<0.02$ in comparison to Non-Ob.

2.3.3 Obesity induces COX-2 expression and increases PGE2 levels in prostate cancer epithelial cells

After establishing that obesity promotes macrophage polarization into TAMs, it was evaluated if PGE2 was responsible for this effect. COX-2 expression as well as PGE2 levels were measured in prostate cancer cells made in response to exposure to sera from obese or non-obese men (**Figure. 2.3**). Results were consistent in LNCaP and LAPC-4 cells: obese conditions increased both COX-2 expression and PGE2 levels when compared to non-obese conditions. In LNCaP cells, COX-2 expression increased by 62% and PGE2 levels were 3.3 times higher. In LAPC-4 cells, there was a threefold increase in COX-2 expression and a 50% increase in PGE2 levels.

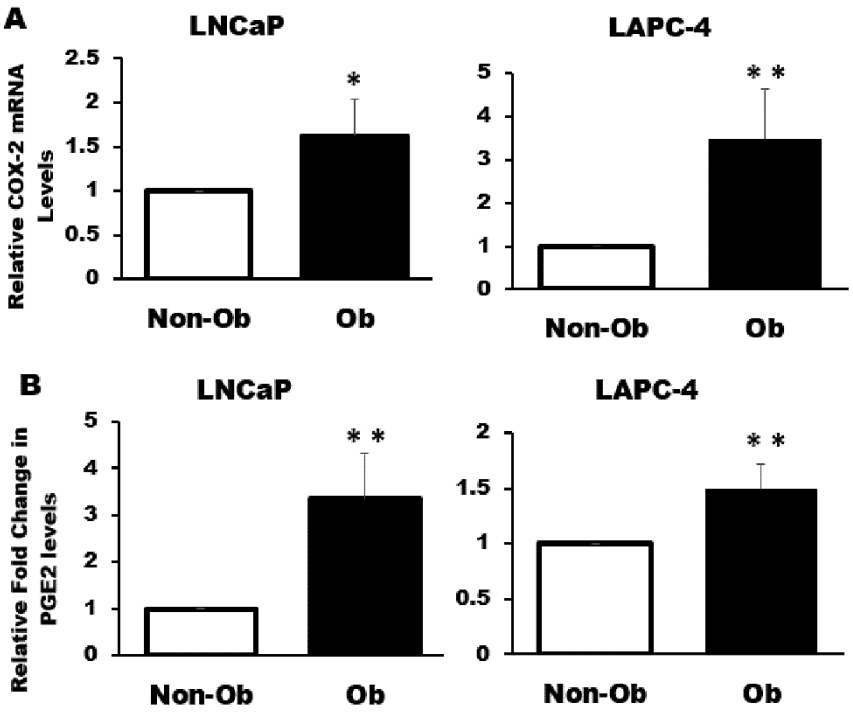

Figure 2.3 Obese-like conditions resulted in significantly higher COX-2 expression (*A*) and PGE2 production (*B*) compared to non-obese.

To determine if prostaglandin-E2 is associated with a TAM phenotype, the expression of the enzyme responsible for its formation has been measured. Prostate cancer cells were treated with sera from obese and non-obese men, COX-2 mRNA levels were determined by qPCR and PGE2 levels were measured by ELISA. *, $p<0.05$ and **, $p<0.02$ in comparison to Non-Ob.

2.3.4 PGE2 may play a role in the polarization of macrophages towards tumor-associated phenotype

To determine if PGE2 is involved in the mechanism by which obese conditions were inducing the TAM polarization, the COX-2 inhibitor celecoxib was utilized. Conditioned media were generated for Ob, Non-Ob and Ob+celecoxib conditions. Compared to the Ob condition, Ob+celecoxib significantly reduced the expression of TGF-beta and IL-10 by 53% and 70% respectively, when treated with LNCaP CM (**Figure 2.4A**). When treated with LAPC-4 CM, celecoxib decreased TGF-beta expression by 29% and Arginase-1 by 44% (**Figure 2.4B**) compared to Ob, but these results were not statistically significant.

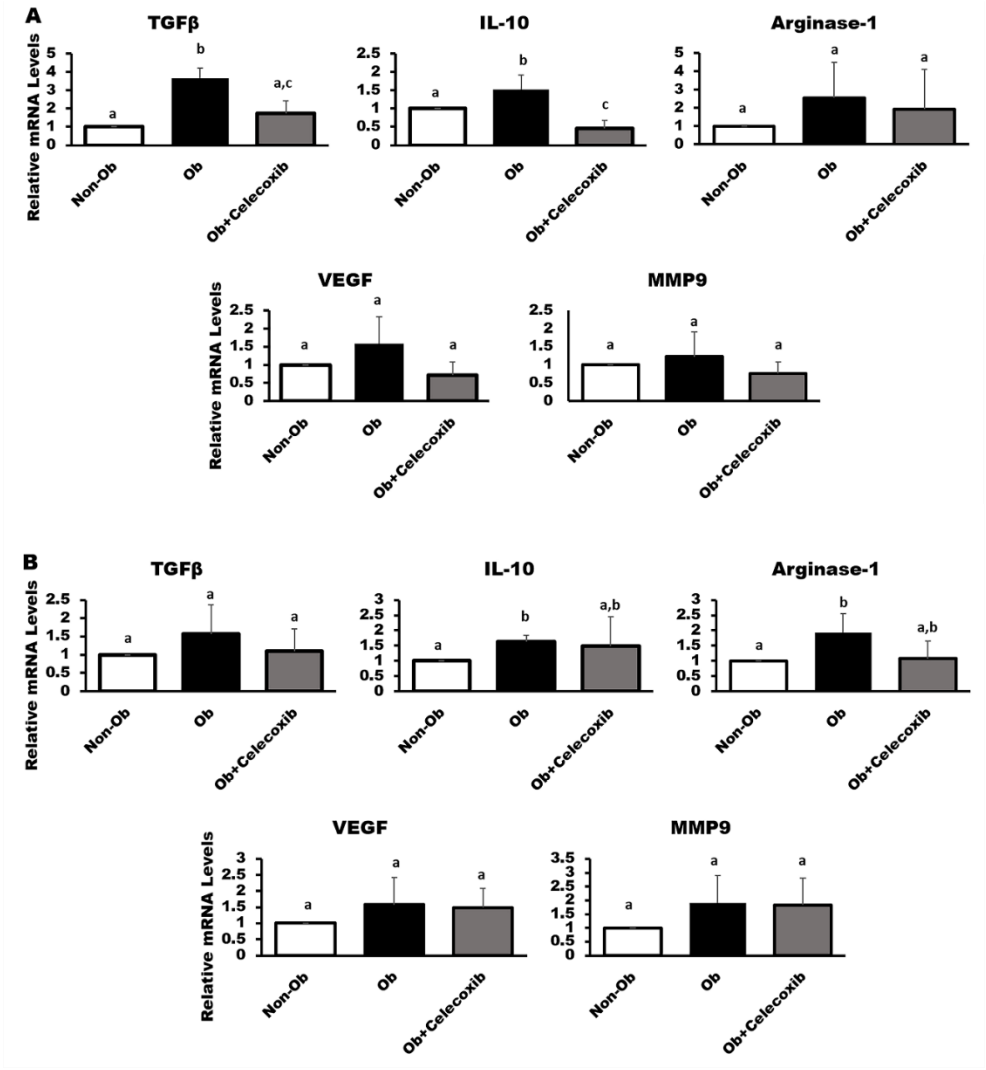

Figure 2.4 Celecoxib reduced obesity-induced expression of TAM markers.

To determine if prostaglandin-E2 induces a TAM-like phenotype in macrophages, macrophages were treated with conditioned media from prostate cancer cells and Celecoxib. The expression of TAM markers (IL-10, TGF-beta, VEGF-A, Arginase-1 and MMP-9) was observed by qPCR after macrophages were exposed to conditioned media from LNCaP (*A*) or LAPC-4 (*B*) cells. *, $p<0.05$ and **, $p<0.02$ in comparison to Non-Ob.

2.4 DISCUSSION

Macrophages are recruited into tumors by local growth factors, inflammatory cytokines, and chemokines. There is increased interest in CCL2 because it is one of the key chemokines involved in the attraction of monocytes and macrophages and it has been found to circulate at higher levels in an obese state (Sartipy, 2003). Previous studies have shown that adipose cells are one of the major contributors of CCL2 (Ito, 2015) (Takahashi, 2003). Given that a higher number of adipose cells are usually present in an obese state and that the prostate gland is surrounded by fat, the objective was to determine if obesity would promote more macrophage infiltration into the prostate tumor. Furthermore, it was determined whether prostate cancer cells themselves could also contribute to the production of CCL2, especially in an obese state. Findings of this study suggest that the tumor microenvironment of an obese individual might recruit a higher concentration of macrophages into the tumor microenvironment than that of a non-obese individual. These results were confirmed *in vitro* and *ex vivo*. It was observed that CCL2 expression is increased by an obesity-induced stimulus, in ASC and LNCaP, but LAPC-4 do not seem equally sensitive to this stimulus. These results were not surprising given that both adipose and cancer cells are known to produce CCL2, however, the difference in the results between the two prostate cancer cell lines, LNCaP and LAPC-4 was not expected. In mice, a higher concentration of TAMs in the obese group compared to non-obese group was found, however, this was only true for mice with high-grade tumors. These results would corroborate previous findings that link high concentration of tumor-associated

macrophages and disease progression (Blando, 2011). CCL2 inhibitors, which are currently under investigation, could be used to test the hypothesis that CCL2 is the main mechanism by which macrophages are recruited into the TME during obesity. These effects could be observed *in vivo* first, and then *in vitro* to determine which cell type is the major contributor of CCL2 that could be driving macrophage infiltration into the tumor. Furthermore, the role of the CCL2 receptor should be investigated.

In this *in vitro* model, it was shown that a tumor-associated phenotype was favored through obesity-induced paracrine signaling. Obese conditions favored the expression of TAM markers in macrophages exposed to obesity-stimulated conditioned media compared to non-obese conditions. The expression of IL-10, TGF-beta and arginase-1 increased under obese conditions when treated with conditioned media from two prostate cancer cell lines. VEGF-A and MMP-9 showed inconsistent expression in macrophages treated with obese and non-obese conditioned media. From the TAM markers analyzed in this study, TGF-beta, IL-10 and Arginase-1 are the most involved in immunosuppression, while VEGF-A and MMP-9 are associated with the metastatic properties of tumor-associated macrophages (Quatromoni, 2012). However, TGF-beta has also been previously linked to metastasis by its involvement in activating epidermal mesenchymal transitions in cancer cells (Scheel, 2011). Further studies should look at the effects of TGF-beta, IL-10 and Arginase-1 on prostate cancer cells and on other cells of the TME, to observe how obesity-related TAMs can drive changes in tumors.

These data also provided evidence that obesity increases prostate cancer cell COX-2 expression and elevated PGE2 production. These results suggest that circulating factors in the sera from obese men induce the production of PGE2, which is overexpressed in many cancer types (Wang, 2010). Some studies have shown that *in vitro*, the addition of PGE2 to stimulated monocytes has shifted the polarization towards an M2 phenotype (Heusinkveld, 2011) (Obermajer, 2011). In this study, it has been shown that inhibition of PGE2 production reversed the effects of obesity on TAM polarization when macrophages were polarized with LNCaP CM; however, the data suggest this is not the only mechanism by which obesity induces TAMs. Some studies have suggested there are other molecules that could be acting synergistically with PGE2 to induce TAM polarization, such as IL-6 which has been shown to be elevated in obesity (Elchaki, 2014). However, future studies will determine if other IL-6 plays a role in obesity-induced polarization of macrophages towards a TAM phenotype. In addition, future studies should be conducted to look how PGE2 induces a TAM phenotype, by looking at the activation of the different PGE2 receptors on the macrophages.

In conclusion, the current study demonstrated that obesity-related circulating factors impact the tumor-microenvironment by promoting macrophage recruitment and polarization of macrophages towards a tumor-associated phenotype in prostate cancer. Elucidating how tumor cells, infiltrated macrophages, and adipose cells interact with each other in the prostate microenvironment will provide new knowledge about prostate cancer progression in obese men. Finally, a better understanding of the impact of obesity on the

polarization process of macrophages towards a tumor-associated phenotype will help to identify possible efficacious and targeted therapies to increase treatment response in obese patients, and ultimately decrease prostate cancer mortality in this population.

Chapter 3: The effects of obesity-induced tumor-associated macrophages on prostate cancer progression

3.1 Introduction

Prostate cancer is the second most common cancer among men, affecting 1 in 7 men in the United States. Although the risk of dying from prostate cancer remains low, the risk of mortality is highly impacted by obesity. This is a significant matter, given that the obesity prevalence in the United States is continuously growing. According to the CDC, in 2015 one third of adults were affected by obesity in the United States. Obesity is known to play an important role in tumorigenesis, it has been linked to the incidence and progression of certain cancers, such as breast, colon and prostate. In prostate cancer specifically, obesity has been linked to a more aggressive disease and worse patient outcomes (Cao, 2016). Compared to their non-obese counterparts, obese prostate cancer patients are more likely to experience high-grade disease and increased risk of recurrence and prostate cancer mortality (Freedland, 2005). While the mechanisms that connect obesity and prostate cancer remain unknown, some studies have shown that peri-prostatic adipose tissue may contribute to the aggressiveness of prostate cancer, due to the low-chronic inflammatory state enriched by the presence of adipose cells and the recruitment of immune cells.

Prostate tumors are usually composed of cancer and stromal cells, including those that get recruited such as circulating immune cells. Immune cells, in particular macrophages, are known to be key players in regulating anti and pro-tumorigenic activities.

Macrophages get recruited, and once in the tumors they can be converted into a tumor-associated phenotype. These changes are driven by signaling molecules coming from cancer cells, with the attempt to create a more favorable environment for tumor growth, and escape the effects of an immunosurveillant environment by inducing TAMs. TAMs are known to drive tumor growth and angiogenesis, which are both associated with a poor prognosis. Some studies have looked at the correlation between high number of TAMs and survival in different forms of cancer. In prostate cancer, high numbers of TAMs have been correlated with worse patient outcome (Lewis, 2006), and to a shorter survival time . Additionally, another study concluded that TAM numbers alone could predict disease-free survival after prostatectomy (Shimura, 2000). This has lead researchers to believe that TAMs could be a viable target for improving outcomes in prostate cancer. However, further research is needed to continue to evaluate this association.

Some of the most important pro-tumorigenic functions of tumor-associated macrophages include the ability of these cells to promote growth, matrix remodeling and angiogenesis, among other functions that favor metastasis (Hanahan,2012). Highly angiogenic tumors provide with increased influx of oxygen and nutrients leading to tumor growth, and with potential exits for migrating cells to travel through the bloodstream increasing the chances of metastasis. TAMs are known to secrete pro-angiogenic factors and cytokines that could lead to blood vessel formation around tumors. One of the main regulators of angiogenesis is vascular endothelial growth factor alpha (VEGFa) and it is expressed by both TAMs and cancer cells. Another way by which macrophages and cancer

cells act together to induce metastasis, is by favoring the degradation of the extracellular matrix (ECM) with the production of matrix metalloproteinases (MMPs). Many studies have focused on the role of TAMs in metastasis and angiogenesis. However, very few have looked at the involvement of TAMs in the regulation of epithelial to mesenchymal transition (EMT). EMT has been linked to tumor progression partially through the regulation of transcription factors such as Snail1 and Twist, and the loss of function of E-cadherin in the cancer epithelial cells (Tania, 2014). TAMs are thought to be implicated in these activities because they are major producers of TGFβ (transforming growth factor-beta) and EGF (epidermal growth factor), molecules that have been known to regulate EMT-related processes. Previous research invariably correlates EMT with more invasive and metastatic tumors; however, it is not clear what the role of TAMs is in this transition.

In this study, the effects of obesity-induced TAMs on prostate cancer progression are investigated by observing how TAMs modulate these activities in the cancer cells. The impact of TAMs on cancer cells was assessed by looking at the invasive capacity, migration and survival ability, as well as the expression of angiogenesis and EMT related markers in prostate cancer cells. Studying these mechanisms further explains how tumor-associated macrophages enhance tumor cell invasion and promote metastatic lesions that worsen patient outcome.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and reagents

Androgen-sensitive prostate cancer cell lines, LNCaP were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). U937 monocytes (ATCC) were converted to macrophages at the time of seeding for experiments by adding 10.0 ng/mL phorbol 12-myristate 13-acetate (TPA) (Sigma) and incubating for 48 hours, as previously described in Chapter 1. U937 cells were used because of their monocytic characteristics which gives them the ability to be differentiated into macrophages. LNCaP and U937 were grown in complete media made with RPMI-1640 (GIBCO Life Technologies) containing 10% fetal bovine serum (FBS), penicillin and streptomycin. Cells were maintained in a 5% (v/v) CO_2 humidified incubator at 37°C. Macrophage colony stimulating factor (M-CSF) was used to induce M2/TAM-like macrophages. These macrophages were used as a positive control for the experiments. M-CSF was purchased from Biolegend.

3.2.2 Serum samples

Sera from male subjects were purchased from Equitech and pooled by BMI category (Obese: BM I≥ 30 kg/m², Non-Obese: BMI < 25 kg/m²).

3.2.3 Conditioned media

Conditioned media (CM) were generated by seeding $2x10^5$ prostate cancer cells or macrophages per well in 6-well plates. For CM generated with prostate cancer cells, cells were serum-starved for 6 hours and then exposed to 2% sera in serum-free media (SFM) for 1 hour. For CM generated from macrophages, cells were serum-starved for 6 hours and then exposed to LNCaP CM or M-CSF for 24 hours to induce macrophage polarization. After exposure, all cells were rinsed with phosphate buffered saline (PBS) and incubated in serum-free media for 24 hours. CM were collected and stored at -20°C for later use. Macrophages resulting from exposure to CM from LNCaP cells exposed to obese or non-obese conditions were designated as Ob and Non-Ob, respectively. Those exposed to M-CSF are designated as M-CSF.

3.2.4 Colony formation (clonogenic) assay

This assay evaluated the ability of the cancer cells to survive and to grow from a single cell into a colony. Prostate cancer cells were seeded and treated with macrophage CM supplemented with 2% FBS. After 10 days, cells were fixed with 95% methanol and stained with 1% crystal violet for qualitative analysis.

3.2.5 Wound healing (scratch) assay

To evaluate the migration ability of cancer cells, a wound healing assay was conducted. LNCaP cells were seeded and allowed to grow to 80% confluence. Subsequently, the cell monolayer was scraped to create a scratch with a sterile pipette tip.

After generating the wound, cells were washed with PBS, and incubated for 24 hours with CM from the macrophages. Images of the plates were acquired at 0 and 24 hours. For each image, the length of the scratch was measured by using Image-Pro software. The migration rate was quantified by measuring five randomly chosen fields for each well at the same location before and after exposure. Percent wound closure was calculated by dividing the difference of measurement at 0h and 24h by the baseline (0h) and multiplying by 100.

3.2.6 Invasion assay

Prostate cancer cell invasive capability was analyzed by using Corning Matrigel invasion chambers (BioCoat). LNCaP cells were seeded on the upper chamber. CM from the macrophages was used in the lower chamber as chemoattractant. CM was supplemented with 2% FBS. After 48 hours, cells that migrated to the lower chamber were fixed with 95% methanol and stained with 1% crystal violet. Images were taken after staining, and cells were counted at five randomly chosen fields for each well. Invasion was further evaluated by using acetic acid as a de-staining solution, and by measuring the absorbance from each of the conditions.

3.2.7 Quantitative PCR (qPCR)

The expression of angiogenesis-related proteins VEGF and HIF-1a, and EMT-related proteins Twist and Snail1, was measured in prostate cancer cells in response to the different conditions explained above. Expression levels were measured by qPCR after 24

hours. RNA extraction, reverse transcription and qPCR were performed as described in Chapter 1.

3.2.8 Statistical analyses

Values are presented as mean ± standard deviation. The difference between two different experimental conditions was measured using Student's *t* test or ANOVA. Significance was set at p value of <0.05.

3.3 RESULTS

3.3.1 Prostate cancer cell survival in response to circulating factors from obesity-stimulated TAMs

To elucidate the potential mechanisms by which obesity may promote prostate cancer progression, the effect of obesity-induced TAMs on prostate cancer cell survival and growth was investigated. LNCaP cells were exposed to CM from macrophages stimulated under obese conditions (Ob), non-obese conditions (Non-Ob), or M-CSF for 10 days. This assay was testing for cancer cell survival in response to exposure to different factors secreted by obesity-induced TAMs. Preliminary results from this assay show that LNCaP exposed to CM from M-CSF treated macrophages had the highest number of colonies out of all three treatments. Secondly, the cells who were under the exposure of obesity-stimulated CM had the most number of colonies. Under obese conditions, LNCaP had 20% more colonies than under non-obese conditions (**Figure 3.1**). These results demonstrate that TAM-associated factors induced by obesity induce higher levels of growth in prostate cancer cells, compared to the non-obese stimulation.

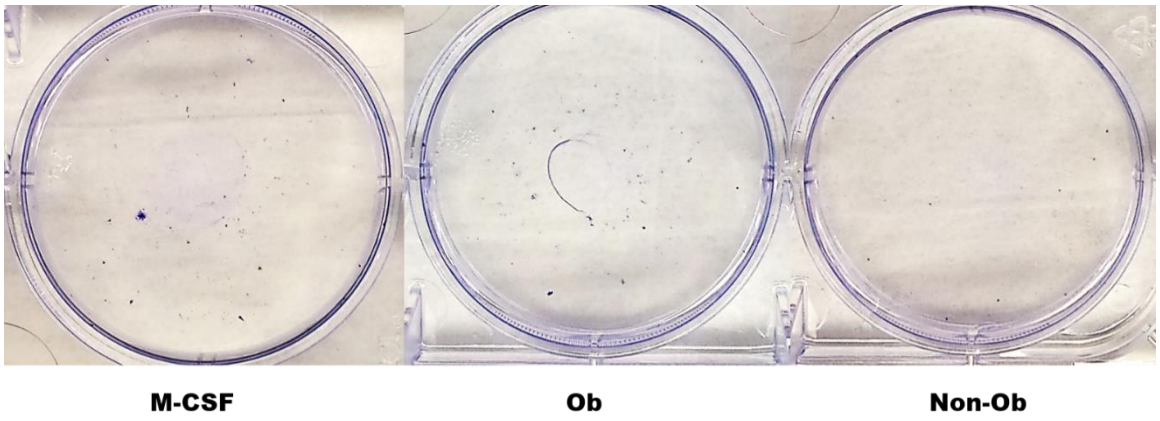

Figure 3.1 Obesity-induced TAMs promoted greater prostate cancer cell survival compared to macrophages stimulated by non-obese conditions.

A colony formation assay was used to assess prostate cancer cell survival. LNCaP were exposed to conditioned media from macrophages previously stimulated in obese conditions (Ob), non-obese conditions (Non-Ob), or MCS-F. After 10 days of exposure, LNCaP colonies were fixed, stained and photographed.

3.3.2 Effects of obesity-stimulated TAMs on the migration capacity of prostate cancer cells

To determine whether the obesity-associated production of growth factors from TAMs could result in enhanced prostate cancer progression, the migration ability of prostate cancer cells, as a result of the impact of macrophage CM, was assessed. A wound healing (scratch assay) was performed with LNCaP cells and CM from macrophages under different stimuli. Forty-eight hours after LNCaP cells were seeded, a scratch was made and cells were exposed to CM from Ob, Non-Ob or M-CSF stimulated macrophages. Pictures were taken at 0h and 24h. Migration rate was assessed by measuring the distance of the scratch before and after treatment. Data is shown as percent wound closure. There was no significant difference in migration rate between conditions (**Figure 3.2**). Since there was no difference between the treatment groups and the control group, these findings suggest that cancer cell migration may not be a hallmark of cancer that is greatly impacted by TAMs.

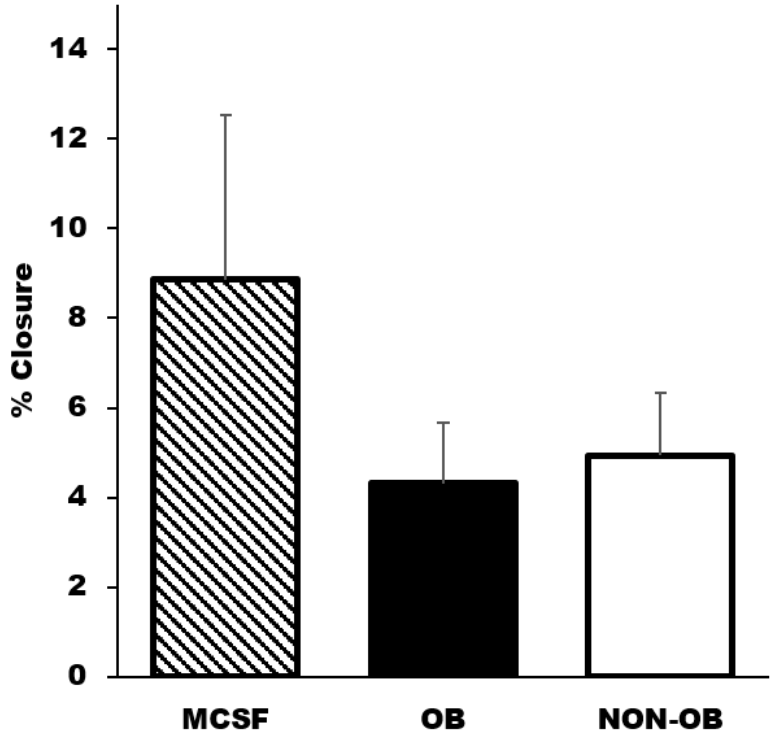

Figure 3.2 Migration rate did not differ between M-CSF, Ob or Non-Ob treatments in LNCaP cells.

To assess migration capability, a wound healing assay was performed in LNCaP exposed to CM from macrophages stimulated under obese or non-obese conditions, or MCSF exposure. Pictures were taken at baseline and 24 hours after the scratch was made. To calculate percent closure, the distance between the cells created by the wound was measured at 0 and 24 hours of exposure to the different conditions.

3.3.3 Invasive potential of prostate cancer cells did not differ between obese and non-obese conditions

One of the main hallmarks of an aggressive tumor, is the capacity of tumor cells to invade. TAMs have been known to induce phenotypical changes in cancer cells that influence their capacity to invade. However, if obesity impacts this process remains unknown. Thus, an invasion assay was conducted to determine whether the secreting factors from obesity-induced TAMs could result in increased invasive abilities of prostate cancer cells. LNCaP cells were seeded at a confluency of 5x10^4 in the inserts of BioCoat Matrigel chamber. CM from macrophages exposed to obese or non-obese conditions, or exposed to M-CSF were used as chemoattractant. The invasive capacity of prostate cancer cells was determined after 48 hours by taking pictures of the different wells, as well as by determining the absorbance of the de-stained solution. Invasion of LNCaP cells did not vary between treatments, as shown by results from this assay (**Figure 3.3**). These results suggest that obesity does not play a role in the invasive potential of prostate cancer cells through the stimulation of TAMs.

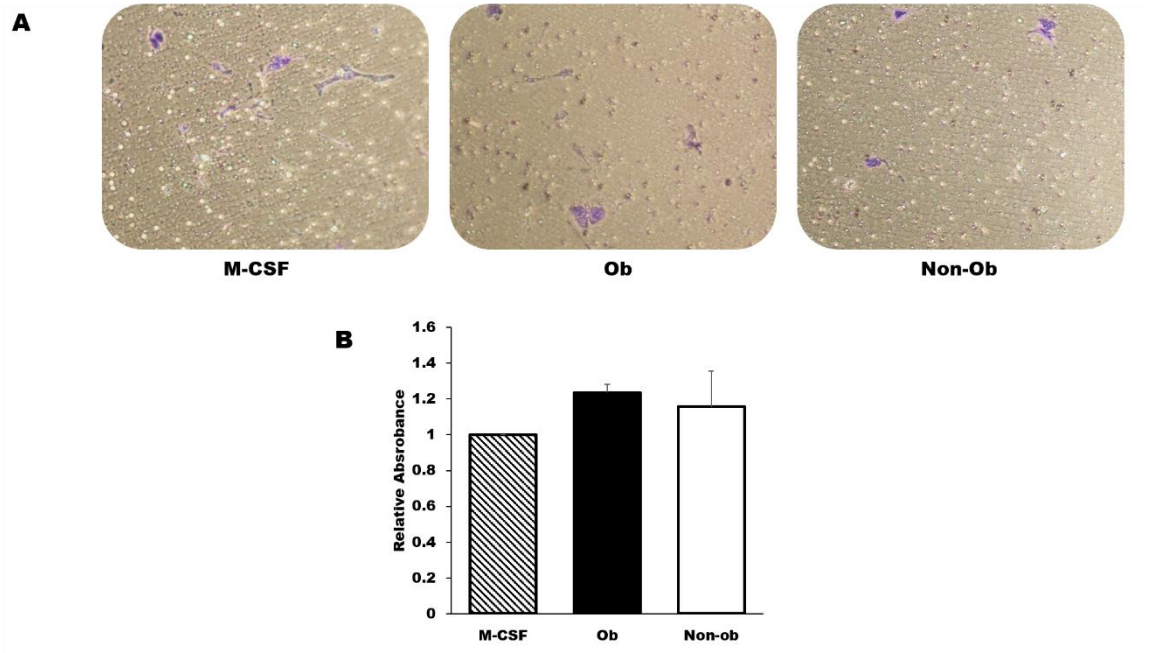

Figure 3.3 LNCaP invasive response to CM from macrophages did not differ between M-CSF, Ob and Non-Ob conditions.

To determine if exposure to obesity-stimulated TAMs increases the invasive capacity of prostate cancer cells, LNCaP were seeded in invasion chambers. CM from macrophages stimulated under obese or non-obese conditions, or under M-CSF exposure were used as a chemoattractant. After 48 hours, cells were fixed and stained, pictures were taken (A), and optical densitometry was measured (B).

3.3.4 Effects of obesity induced TAMs on angiogenesis and EMT

Angiogenesis and EMT have been implicated in making cancer more aggressive and metastatic. To determine if obesity-induced TAMs increased expression of angiogenesis and EMT-related proteins, LNCaP were exposed to macrophage CM for 24 hours. mRNA levels of HIF-1a, VEGFa, Snail1 and Twist were measured after exposure. HIF-1a and VEGFa are some of the main proteins implicated in tumor vascularization, and Snail1 and Twist are transcription factors critical for the activation of EMT.

Results show that expression levels of VEGFa, Snail1 and Twist were significantly greater in the M-CSF group compared to Ob and Non-Ob. However, there was no significant difference in expression levels of this genes in the Ob compared to the Non-Ob treatment (**Figure 3.4**). These results suggest that obesity-induced TAMs do not have the same effect as M-CSF-induced TAMs in the expression of EMT and angiogenic proteins.

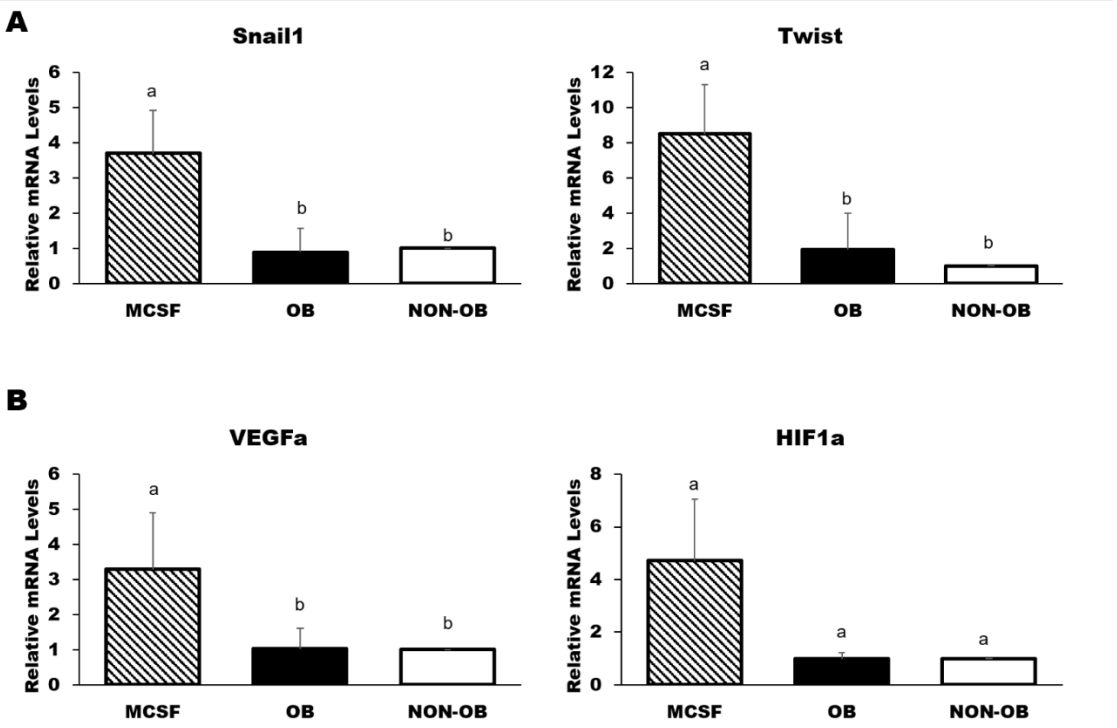

Figure 3.4 Expression levels of HIF-1a, VEGFa, Snail1 and Twist did not differ between Ob and Non-Ob conditions.

mRNA levels of HIF-1a, VEGFa, Snail1 and Twist were detected by qPCR after LNCaP cells were exposed to CM from macrophages for 24 hours. M-CSF conditions increased the expression of Snail1, Twist and VEGFa in comparison to the other treatment groups. CM from obesity-stimulated TAMs did not have an effect on the expression of HIF1a, VEGFa, Snail1 and Twist in LNCaP, compared to CM generated under non-obese conditions.

3.4 DISCUSSION

Due to the alarmingly increasing rate of obesity, it is of critical importance to expand the understanding of the role of obesity in cancer development and progression. In prostate cancer, obesity has shown to be correlated with aggressive disease and to increase patient mortality. Even though the mechanisms connecting obesity and prostate cancer progression remain unclear, there is evidence suggesting that obesity-induced infiltration of non-cancer cell to the tumor, such as macrophages, may play a role in driving the tumor towards an invasive and metastatic phenotype.

Cancer cells from aggressive tumors have the ability to proliferate, migrate, invade and induce tumor vascularization. TAMs, aside of helping cancer cells evade immune surveillance, can provide cancer cells with the growth factors and cytokines necessary to induce the hallmarks of cancer that result in aggressive disease.

Data presented in chapter one suggests that obesity plays a role in macrophage infiltration into tumors, and in macrophage polarization towards a tumor-associate subtype with the collaboration of cancer cells. However, the role of obesity-induced TAMs on the promotion of prostate cancer aggressiveness remained unknown. In this study, it has been shown that factors secreted by obesity-induced TAMs may influence cancer cell survival, as evidenced by the results of the clonogenic assay. Preliminary results show that obesity-induced TAMs induce changes in prostate cancer cells that could lead to tumor growth, but do not support the hypothesis about promoting invasion and metastasis.

Previous studies have shown that TAMs are involved in metastatic processes by producing VEGFa and inducing its production on cancer cells as well. There is some evidence, as well, that TAMs could be implicated in the induction of EMT; however, not enough research has been done in this area. In this study, it was found that obesity-induced TAMs do not have an effect on the expression of the EMT-related transcription factors Snail1 and Twist. Overexpression of Snail1 and Twist has been linked to tumor progression and a worse prognosis in prostate cancer patients, at least partially due to the repression of E-cadherin. Loss of E-cadherin and increased vimentin have been shown to be hallmarks of EMT, leading to changes in the morphology of cancer cells that traditionally result in metastasis (Tania, 2014). Future studies should look at the effects of obesity-induced TAMs on e-cadherin status.

Obesity-stimulated TAMs appear to have no effect on migration and invasion of cancer cells as demonstrated by the lack of effect on the wound healing and invasion assays performed in this study. These results may indicate that obesity does not play a role in enhancing the abilities of cancer cells to migrate and invade through the stimulation of TAMs. However, that does not mean that obesity cannot promote these effects directly on the cancer cells potentially through obesity-associated circulating factors, as shown in previous studies done in this lab. Additionally, in migration and invasion, there was no difference between the M-CSF conditions, which was used as a positive control, and the Ob and Non-Ob conditions. These results could indicate that TAMs do not have a notable effect in changing the capacities of prostate cancer cells to migrate and invade. These were

surprising results given that these are some of the main hallmarks of cancer that TAMs are typically involved in modulating.

In future studies, the effects of obesity-induced TAMs in non-cancer prostate epithelial cells could be determined, to assess if there is an induction of tumorigenic activities in early stages.

In summary, this study provides suggestive evidence that obesity-related factors stimulate TAMs to induce growth and survival in prostate cancer cells. Further research is needed to continue to determine if obesity-induced TAMs promote prostate cancer progression. The use of co-cultures involving prostate cancer cells and macrophages, instead of the use of CM, could potentially have a different effect on the cancer cells. Analyzing the different factors and cytokines that are being secreted by macrophages in response to the different stimulations (Ob, Non-Ob, M-CSF) could help to identify what effects they can have on the cancer cells. Future studies should continue to address the implications of obesity in programming the tumor microenvironment and to further investigate the effects of different subtypes of macrophages on cancer cells.

Chapter 4: Rapamycin selectively targets TAM-like macrophages

4.1 INTRODUCTION

Recent studies have shown that the composition of the tumor microenvironment TME, particularly the presence of TAMs induce metastasis in certain types of cancer (Lewis, 2006). This is highly relevant given that macrophages form most of the inflammatory cell types that infiltrate into the TME (Bingle, 2002). Some studies, have indicated that high numbers of TAMs are positively correlated with advanced disease states and poor prognoses in prostate cancer (Lissbrant, 2000).

Macrophage subtypes vary according to the different signals they receive from the environment in which they are located. The classically activated M1, and the alternatively activated M2 subtypes, are located at the end of the spectrum of macrophage subtypes. While the M1 phenotype exerts pro-inflammatory activity, the M2 phenotype is anti-inflammatory and immunosuppressive (Biswas, 2010). TAMs have been recognized as mostly M2-like, and have been implicated in disease progression due to their ability to induce immunosuppression through the release of cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-beta). Additionally, they are known to promote metastasis by releasing vascular endothelial growth factor alpha (VEGFa) and matrix metallopeptidase 9 (MMP9) (Lewis and Pollard 2006). Results from Chapter 1 indicated that under obese conditions, signaling from prostate cancer cells induced macrophages to become TAMs, as shown by the increased expression of TAM markers such as IL-10,

TGF-beta and Arginase-1 in such cells. Another way to distinguish between macrophage subtypes is by looking at the different markers expressed in the surface of the cell in each of the subtype. For example, CD206 (mannose receptor) is expressed in M2, but not M1 macrophages. Therefore, CD206 is a useful marker to distinguish between the two subtypes. Knowing what type of macrophage population is present in the tumor could aid in designing targeted therapies to improve patient outcome.

A critical component to improve patient outcome will be to identify a potential therapeutic target to limit polarization and proliferation of these tumor-promoting macrophages. Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, has been used for several decades in transplant patients to prevent organ transplant rejection because of its ability to modulate immune responses (Snell, 2001) (Shapiro, 2000). In recent years, rapamycin has been shown to be an effective disease suppressor in some types of cancer due to its antiproliferative effects (Huang, 2004). Additionally, there is growing evidence that mTOR is critical for the activation and survival of certain immune cells, and is especially important in macrophage polarization and stabilization, particularly for M2 macrophages (Weichhart, 2009) (Mercalli, 2013).

Studies that have looked at targeting mTOR signaling for prostate cancer therapy have focused on cancer cell proliferation since the PI3K/Akt/mTOR signaling pathway is the second most commonly altered pathway in prostate cancer. This is true particularly in PTEN-negative tumors, which constitute about 60 percent of prostate tumors (Kremer, 2006). The alteration of this pathway results in hyperactivation of the mammalian target of

rapamycin (mTOR), particularly in advanced prostate cancer, making mTOR a therapeutic target with outstanding potential. Nevertheless, mTOR inhibition has not always been successful when targeting cancer cells. Consequently, it was proposed to target cells from the tumor microenvironment instead that are known to drive cancer progression considering that these cells may be more susceptible to rapamycin treatment. The role of mTOR in the modulation of the prostate tumor microenvironment, specifically in the activation of a tumorigenic phenotype in macrophages, is not fully understood and should be further elucidated.

The overall objective of this study was to investigate if rapamycin could be used for targeting TAMs in the prostate tumor microenvironment with the ultimate goal being to prevent metastasis and disease progression Since previous studies have shown that mTOR signaling is important in the differentiation of M2, it was hypothesized that mTOR signaling is necessary for the differentiation of TAMs, consequently, rapamycin will selectively target the survival of TAMs. To address this hypothesis, an *in vitro* model of macrophage polarization was used.

4.2 Materials and Methods

4.2.1 Cell lines and reagents

U937 monocytes and androgen-sensitive prostate cancer cell line, LNCaP, were used. LNCaP and U937 cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). Prostate cancer and monocytic cell lines were grown in RPMI-1640 (GIBCO Life Technologies) containing penicillin and streptomycin plus 10 percent fetal bovine serum (FBS) cell lines were maintained in a 5 percent (v/v) CO_2 humidified incubator at 37°C. Rapamycin was purchased from Sigma-Aldrich.

4.2.2 Macrophage differentiation and polarization

Monocytes were differentiated into macrophages at the time of seeding for experiments by adding 10.0 ng/mL phorbol 12-myristate 12-acetate (TPA) (Sigma) and incubating for 48 hours.

Macrophages were polarized into TAM-like by being exposed to conditioned media (CM) from LNCaP, as explained in Chapter 2. M1-like macrophages were exposed to CM from prostate cancer that did not receive additional stimulation.

4.2.3 Rapamycin treatment

Macrophages were treated with low (1 nM) and high (10 nM) doses of rapamycin. Different doses were used to compare the effects of the drug in a dose response manner.

Rapamycin was prepared in 1 and 10 uM stock solutions using DMSO. Vehicle controls of DMSO were tested in parallel with the cells treated with rapamycin.

4.2.4 CD206 detection

CD206 was used to determine if the TAM-like macrophages from this model present M2 characteristics. Cell surface marker CD206 was detected using flow cytometry. Macrophages were polarized, and cultured in the presence or absence of rapamycin. After 48 hours, cells were collected, washed twice using harvesting buffer (1X PBS, 3% BSA, 1% Na Azide), and counted. After resuspending cells to a concentration of 1x10^6 cells/ml, cells were incubated with anti-CD206 (abCam) in the dark for 1 hour at 4°C, and FITC secondary (Jackson ImmunoResearch) in the same conditions as the primary antibody. Fluorescence was detected using an Accuri C6 flow cytometer. Analysis was done using FlowJo to determine the percentage of CD206 positive cells in each condition.

4.2.5 Cell viability assay

Cell viability was determined by assessing cell metabolic activity measured by MTT. U937 cells were seeded in 200 µl complete RPMI at a density of $5x10^3$ cells per well in 96-well plates with 10 ng/ml of TPA. Forty-eight hours after seeding and exposure to TPA, the cells were washed and then exposed to conditioned media. After 24 hours of exposure to conditioned media, the media was aspirated, and cells were washed and then treated with 0nM, 1nM, and 10nM Rapamycin in 200 µl RPMI. After 48 or 96 hours of treatment, 20 µl MTT solution (5 mg/ml in PBS) was added. After 3 hours of incubation

at 37°C, the media were removed and 100 µl DMSO was added to each well to solubilize MTT crystals. Absorbance was read at 570 nm on a BioTek Synergy 2 Multi-Mode Microplate Reader. Relative growth inhibition was calculated by dividing each absorbance value by the absorbance for cells grown in the 0 nm Rapamycin treatment, multiplying by 100, and then subtracting from 100.

4.2.6 Apoptosis analysis

Apoptosis was determined using Annexin V and propidium iodide (PI) staining. Macrophages were polarized and cultured with or without low or high dose rapamycin. After 48 hours, Annexin V and PI staining was done with the FITC Annexin V apoptosis detection kit with PI according to manufacturer's instructions (Biolegend). Fluorescence was detected using an Accuri C6 flow cytometer. Analysis was done using FlowJo to determine the percentage of apoptotic cells in each condition.

4.2.7 Statistical analyses

Values are presented as mean ± standard deviation. The difference between two different experimental conditions was measured using the Student's *t* test. Significance was set at a p value of <0.05.

4.3 RESULTS

4.3.1 CD206 expression in M1 and TAM-like macrophages

To determine if the TAM-like macrophages generated in this model exerted an M2 phenotype, CD206 expression was determined by flow cytometry. CD206, mannose receptor, is up-regulated in M2 macrophages to direct wound healing-related activities. This cell surface protein serves as a useful marker to distinguish between M1 and M2 macrophages, because it is not expressed in M1. In this experiment, macrophages were polarized under different stimuli, and the resulting macrophage phenotypes were determined by staining the cells with anti-CD206. Flow cytometry was used to quantify the number of CD206 positive cells. TAM-like macrophages exerted an M2 phenotype, as determined by the elevated expression of CD206 compared to M1-like macrophages (**Figure 4.1**).

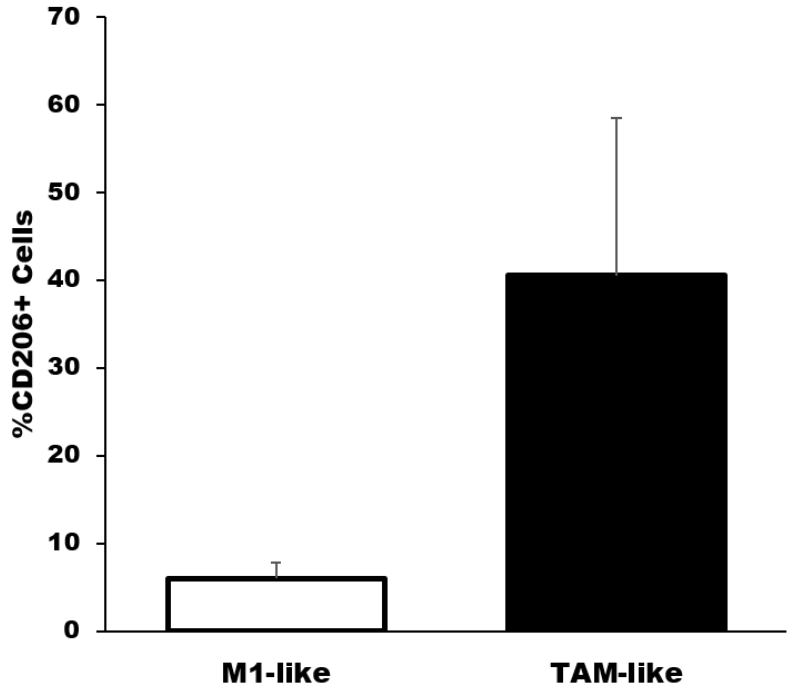

Figure 4.1. A higher number of TAM-like macrophages expressed CD206 compared to M1-like macrophages.

CD206 expression was measured by flow cytometry after macrophages were differentiated into different subtypes. Data is presented as percentage of CD206 positive cells. Preliminary results show that a higher number of cells were CD206+ in the TAM like population compared to M1-like population.

4.3.2 Rapamycin selectively targets TAMs

To determine if rapamycin would target TAM-like macrophages preferentially, viability was measured in M1 and TAM-like macrophages after being treated with rapamycin for 48 hours. Cell viability was measured by conducting an MTT assay, and growth inhibition was calculated. TAM-like macrophages treated with rapamycin exhibited three times more growth inhibition in comparison to M1-like at 1nM rapamycin treatment (**Figure 4.2**). At 10 nM, growth was also greatly inhibited in TAM-like macrophages compared to M1-like, but results were not statistically significant.

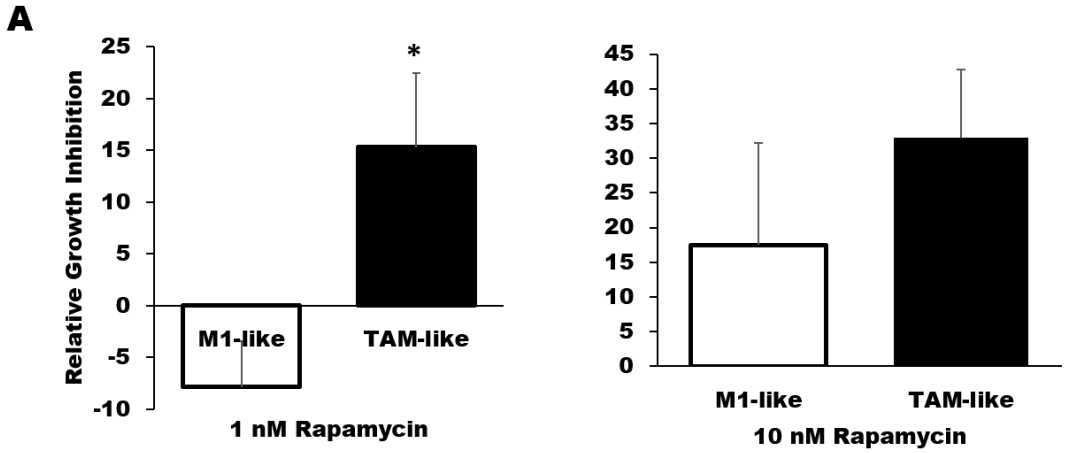

Figure 4.2 mTOR inhibition selectively decreased TAM-like viability.

An MTT assay was conducted to determine if rapamycin had an effect on the viability of M1 and TAM-like macrophages. TAM-like macrophages exhibited a significant increase in growth inhibition in comparison to M1-like macrophages at 1 nM rapamycin ($P<0.001$) (A).

4.3.3. Rapamycin may decrease viability of TAM-like macrophages by inducing apoptosis

To determine the mechanism by which rapamycin affects cell viability, apoptosis was determined by Annexin V and propidium iodide (PI) staining. Flow cytometry analysis was used to measure apoptotic cells at 48 hours. Preliminary results suggest that a higher percentage of TAM-like macrophages are undergoing apoptosis, compared to M1-like macrophages (**Figure 4.3**). However, this difference is only observed in the 10nM treatment.

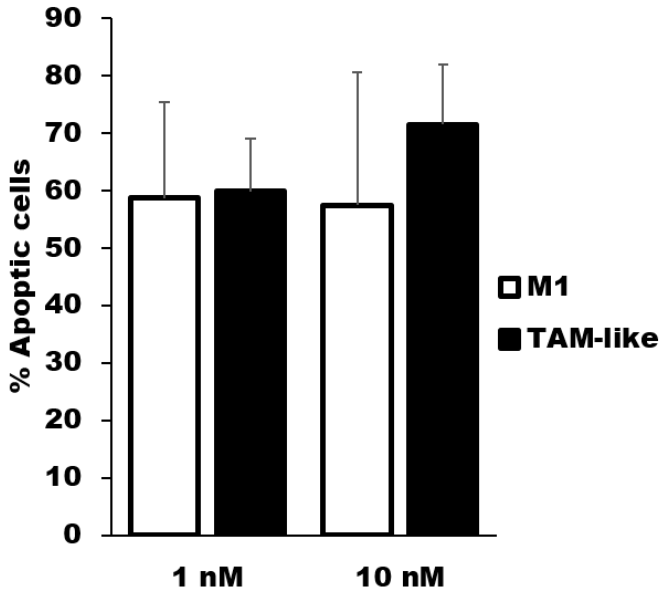

Figure 4.3 Rapamycin induced a higher percentage of apoptotic cells in TAM-like macrophages compared to M1-like macrophages.

Apoptosis was determined in M1 and TAM-like macrophages treated with low and high dose rapamycin, by measuring the percentage of apoptotic cells in each population by flow cytometry. Cells were stained with Annexin V and propidium iodide to determine if apoptosis was occurring. Preliminary results show that the percentage of apoptotic cells is higher in TAM-like macrophages when treated at a higher dose.

4.4. DISCUSSION

TAM-like macrophages exhibit M2 properties that could make them possible targets of rapamycin. The expression of the cell-surface protein CD206, also known as mannose receptor, is one of the main characteristics of M2 macrophages, thus it was used to detect an M2 phenotype. In this study, TAM-like macrophages had higher percentage of CD206 positive cells, compared to M1-like macrophages. These results confirm that the TAM-like macrophages developed in this model display M2 properties, and therefore, can be targeted by rapamycin.

Rapamycin treatment, at 1 nM, significantly reduced cell viability in TAMs in comparison to M1. Although the molecular mechanism behind mTOR regulation of M2/TAM-like macrophages has not been identified, these results are consistent with prior studies demonstrating that mTOR inhibition plays a role in the survival of M2-like macrophages. Interestingly, this study showed that, even at low doses (1 nM), rapamycin reduced cell viability in TAMs. Similar experiments should be conducted with other mTOR inhibitors to see if this result is replicated.

The effects of rapamycin on apoptosis appear to differ between M1 and TAM-like macrophages. Induction of apoptosis could potentially be the mechanism by which treatment of rapamycin decreases TAM survival compared to M1 macrophages. However, more experiments are needed to further evaluate this mechanism. Such studies should be conducted to determine why this effect is observed mostly in TAMs and not M1s. It would also be important to further investigate the activation of PI3K/Akt/mTOR signaling

pathway in different subtypes of macrophages. However, it is likely that apoptosis is not the solely mechanism by which rapamycin targets TAMs. Surprisingly, the viability of TAM-like was mostly decreased when treated with low rapamycin, but apoptosis was only induced when rapamycin was used at higher doses. Some studies have shown that in cancer cells, the inhibition of mTOR by rapamycin is known to result in apoptosis, as well as cell cycle arrest (Peponi, 2015). Future studies should look at cell cycle arrest because that could be the mechanism by which rapamycin is decreasing viability at low doses in TAM-like macrophages.

As previously mentioned, most cancers are primarily populated by TAMs that are M2-like and promote growth. Some studies have shown that when the presence of M1 is boosted, tumor growth is inhibited (Ruffell, 2015)(Mills, 1992). Considering the findings in this study, future studies should investigate the effect of rapamycin on macrophage polarization from an M2 to an M1-like phenotype. If such polarization occurs, then mTOR inhibition could be a great tool to repopulate tumors with M1 macrophages and potentially lead to growth inhibition. Moreover, mTOR inhibition could also make tumors more immunogenic and thus responsive to other immunotherapies.

mTOR inhibitors have shown promising results in blocking tumor growth and metastasis (Xie, 2016). Nevertheless, most of them seem to only be effective in high doses. Results from this study suggest that mTOR inhibition could have effects on the immune cells even with low doses of rapamycin, and therefore have fewer detrimental side effects for patients.

Results from this study suggest that the mTOR pathway is involved in the survival of TAMs. By elucidating that mTOR could be involved in the survival of TAMs, this study offers a novel mechanistic approach to treat prostate cancer. Future directions of this study include looking at the effects of other mTOR inhibitors on TAMs, and determining the mechanisms behind rapamycin's preferential selectivity on M2 compared to M1 macrophages. Lastly, it would be equally important to investigate if rapamycin induces re-polarization of TAMs back to M1 macrophages.

Chapter 5: Concluding Remarks

5.1 CONCLUSIONS

5.1.1 Chapter 2: Obesity regulates the macrophage population in the prostate tumor microenvironment

Obesity is associated with a greater risk of prostate cancer mortality. However, the mechanisms connecting obesity to the progression of prostate cancer remain unknown. To provide a better understanding of the mechanism by which obesity confers a more aggressive disease, the effects of obesity on the prostate tumor microenvironment were determined in this study. The main focus of this study was to determine the impact of obesity on macrophage recruitment and TAM polarization in the prostate tumor microenvironment. It was hypothesized that obesity-associated circulating factors contribute to the recruitment and polarization of TAMs, resulting in aggressive tumors. To test this hypothesis, an *in vitro* model was used. With the objective to mimic the tumor microenvironment, this model included different cell lines including adipose stromal cells, macrophages and prostate cancer cells. Adipose stromal cells were used to look at the contributions of these cells in tumors, given that they are found in abundance in the prostate stroma and known to produce pro-inflammatory factors. Macrophages were obtained from U937 monocytic cells. U937 cells were used because of their ability to differentiate into macrophages. This cell line has been widely used in research while trying to investigate differentiation processes of monocytes and macrophages. As for the prostate cancer cells,

LNCaP and LAPC-4 cells were used. All the prostate cancer cell lines that were used are androgen responsive, but vary slightly in invasive capacity and aggressiveness. Different cancer cell lines were used to evaluate if responses would vary from one cell line to another. To replicate an obese or non-obese state, serum collected from men under different BMI categories was used. Categories were assigned as obese for men with BMI $\geq$ 30 and non-obese for men with BMI < 25 kg/m^2.

In this study, it was observed that macrophage and TAM recruitment, both *in vitro* and *ex vivo,* increased under obese conditions compared to non-obese conditions. However, in mice tumor tissue, TAM infiltration was only observed in mice with high-grade tumors. These results coincide with previous studies showing that TAM numbers are correlated with tumor grade. Additionally, CCL2 expression was induced in prostate cancer cells and adipose stromal cells by exposure of obese sera. CCL2 has been established as one of the main macrophage-recruiting chemokines. In this model, it was interesting to see that cancer cells could potentially recruit macrophages by producing CCL2, and that this process was enhanced by obese conditions.

When looking at TAM polarization, it was found that obese conditions increased mRNA levels of TAM markers in macrophages through paracrine signaling. Factors in the CM from prostate cancer cells, produced in response to exposure from sera of obese men, induced a tumor-associated phenotype in macrophages. The expression of IL-10, Arginase-1 and TGF-beta was significantly increased by obese conditions. These proteins are associated with the M2/TAM subtype of macrophages, and are known to be involved in

immune suppression and cancer progression. Results regarding the expression of TAM markers varied depending on the cell line that was used for generating conditioned media. LNCaP and LAPC-4 CM did not have the same effect on macrophages. PGE-2 is one of the main drivers of polarization of macrophages into TAMs, and it is usually upregulated in the obese state. Therefore, it was hypothesized that PGE-2 could be involved in obesity-induced TAM polarization. To determine if circulating factors in obesity induce PGE-2 production, COX-2 expression and PGE-2 levels were measured in prostate cancer cells after sera exposure. Circulating factors from the obese sera induced COX-2 expression and PGE-2 production in LNCaP and LAPC-4 cells. However, to determine if PGE-2 was responsible for the obesity-induced tumor-associated phenotype in macrophages, PGE-2 production was blocked by using celecoxib, a COX-2 inhibitor. COX-2 inhibition resulted in lower expression of obesity-induced TAM markers. The expression of IL-10, Arginase-1 and TGF-beta decreased when obesity-induced TAMs where pre-treated with celecoxib. These data suggest that obesity may promote macrophage infiltration into the prostate tumor microenvironment, and induces TAM polarization through the COX-2/PGE2 pathway. The effects of PGE2 on TAM polarization were assessed because PGE2 can drive polarization of macrophages towards a TAM phenotype and it is seen at high levels in obesity (Bowers, 2015). Consequently, the next step should be to test whether PGE-2 inhibition blocks TAM presence in the prostate TME of obese transgenic mice. Additionally, the effects of PGE2 signaling on macrophages should be determined.

New knowledge about the role of the tumor microenvironment on prostate cancer progression in obese men was obtained by investigating the relationship between cancer, immune and adipose cells, and how they interact with each other in the tumor. In addition, based on this information a basis for identifying targeted therapies towards obesity-induced TAMs has been established, with the aim to increase response to treatment and decrease prostate cancer specific mortality in obese prostate cancer patients. In conclusion, it was observed that obesity-related circulating factors influence the prostate tumor microenvironment leading to the recruitment of macrophages and the conversion of them into a tumor-associated phenotype. Since this phenotype is known to promote tumor growth and metastasis, the effects of TAMs on cancer cells were investigated in the second study.

5.1.2 Chapter 3: The effects of obesity-induced tumor-associated macrophages on prostate cancer progression

There is strong evidence showing TAMs can regulate tumor development, growth and progression to a metastatic disease. TAMs contribute to cancer progression by enhancing invasive capacity through tissue remodeling, promoting vessel formation through regulation of angiogenic factors and increasing survival of cancer cells through down regulation of immune function (Ruffell, 2012). Clinical studies have found that high numbers of tumor-associated macrophages in tumors are significantly correlated to poor patient prognosis in different cancer types, including prostate cancer (Bingle, 2002). Therefore, this group has been conducting molecular and translational studies to better

understand the molecular mechanisms underlying the relationship between TAMs and cancer cells in the tumor microenvironment.

This study was designed to determine if obesity-induced TAMs from the first study impacted the behavior of prostate cancer cells by inducing a more invasive and metastatic phenotype. In this study; migration, invasion, survival, and the expression of angiogenic and EMT markers were investigated in prostate cancer cells in response to obesity-stimulated TAM CM. Preliminary results of this study showed there was a small increase in survival of LNCaP under M-CSF and obesity-stimulated CM. There was no effect of obesity-stimulated TAMs in changing the migratory and invasive capacity of LNCaP, compared to non-obese or M-CSF stimulation. These results suggest that in this model, TAM-like cells did not have an impact on prostate cancer cells with regards to these two tumorigenic activities. Similarly, the expression of VEGFa, HIF-1a, Snail1 and Twist did not differ between the obese and non-obese conditions. However, the expression of VEGFa, Snail1 and Twist significantly increased in LNCaP exposed to M-CSF conditions, compared to the obese and non-obese conditions. These data suggest that TAM-like macrophages do affect the expression of these markers in the cancer cells, but it also suggests that obesity-stimulation may not be sufficient for macrophages to function as TAMs. In the first study, it was observed that stimulation from factors related to obesity induced a TAM subtype in macrophages, as determined by the increased expression of TGF-beta and IL-10 when macrophages were exposed to LNCaP CM. It is important to note that most of these changes could be induced by the increase of TGF-beta production

of macrophages, but since LNCaP cells do not respond to TGF-beta signaling, the effects of obesity-induced TAMs on prostate cancer cells should be looked at using a different cell line. Further studies should test these effects, and test if TGF-beta is one of the main mechanisms by which obesity-associated TAMs can promote an aggressive phenotype in prostate cancer cells. Similarly, the effects of IL-10 should be explored. IL-10 is a key mediator of immune suppression, therefore the impact of obesity-stimulated TAMs on immune cells, such as T-cells, should be further explored.

The objective of this study was to determine if TAMs are potential therapeutic targets in prostate cancer patients, particularly in those who are obese. Further research is needed to determine the effects of obesity-induced TAMs in prostate cancer progression.

5.1.3 Chapter 4: Rapamycin selectively targets TAM-like macrophages

Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, has been used for several decades in transplant patients, and in the last several years has been shown to be an effective disease suppressor in certain cancer types. Intriguingly, mTOR has been shown to be especially important for M2 polarization and stabilization, and since TAMs are M2-like, mTOR inhibition could affect TAMs as well. Based upon published data and preliminary studies, it was hypothesized that rapamycin selectively targets TAM-like macrophages and provides a survival benefit to prostate cancer patients. To address this hypothesis, an *in vitro* model of macrophage polarization was used.

The cell surface marker CD206, which is associated with M2/TAM, was measured to determine macrophage phenotype. Macrophages were characterized by looking at

CD206 expression in macrophages polarized through different conditions. TAM-like macrophages presented a higher percentage of CD206 positive cells in its population, compared to the M1-like subtype. These results suggest that in this model, TAM-like macrophages are M2-like and thus could be targeted by rapamycin. Cell viability and apoptosis were analyzed to identify the effects of rapamycin on the different macrophage subtypes. Results of these assays showed that rapamycin inhibited cell viability in TAM-like macrophages selectively. The difference in growth inhibition by rapamycin was significantly different between M1-like and TAM-like macrophages when treated with low doses (1 nM) of the drug, but it did not reach significance when at 10 nM. A potential explanation for a lack of significance in the cells treated with 10 nM of rapamycin, is that the dose is so high that rapamycin is targeting both macrophages subtypes equally. Other findings from this study include preliminary results suggesting that one of the mechanisms by which rapamycin may be targeting TAMs, is by inducing apoptosis in this macrophage subtype. As demonstrated by flow analysis, rapamycin induces higher levels of apoptosis in TAM-like macrophages compared to M1-macrophages. Intriguingly, this difference looks more prominent only when the cells were treated with the higher dose of rapamycin (10 nM). Although it was expected that higher doses of rapamycin would induce higher apoptosis, these results are not consistent with the results from the viability assay, where growth was being inhibited only at 1 nM. Research studies that have looked at the effect of rapamycin on cancer cells have seen that rapamycin can inhibit tumor growth by inducing apoptosis and cell cycle arrest (Xie, 2016). A potential explanation for the

discrepancy between the cell viability and the apoptosis data, is that rapamycin could be inducing cell cycle arrest in TAM-like macrophages at low doses, and apoptosis at high doses. To test this hypothesis, cell cycle status in these cells will be determined. To look at the mechanisms by which rapamycin targets TAMs, proteins involved in cell cycle and apoptosis, such as Cyclin D1 expression and PARP cleavage, should be evaluated.

In conclusion, this study provided new and critical knowledge regarding mTOR regulation of immune responses, as well as new insights about mTOR inhibitors with respect to treating cancer. The scientific significance of this study was based on a model that allowed us to determine if mTOR plays a role in immune cell differentiation, specifically in the macrophages. Additionally, this study could be the basis of important clinical implications by providing evidence that rapamycin could be used to target tumor associated macrophages in the tumor microenvironment and potentially use it as an effective therapeutic target to limit prostate cancer progression and patient mortality. The underlying challenges this research addressed are, to develop strategies to prevent progression to lethal prostate cancer by targeting TAMs in the tumor microenvironment, and to identify effective treatments and address mechanisms of resistance for men with high-risk or metastatic prostate cancer. This study was designed to be applied as a personalized medicine approach where it will be possible to identify novel therapeutic targets and approaches to improve treatment efficacy and reduce mortality rates in prostate cancer patients, particularly in those at high risk.

5.2 FUTURE DIRECTIONS

These set of studies presented have demonstrated evidence suggesting that obesity may promote prostate cancer progression by inducing a programming in the tumor microenvironment that involves macrophage recruitment and polarization into a TAM phenotype. However, these studies were done in *in vitro* models, and these do not fully recapitulate what happens *in vivo*. To discover the biological relevance of the data presented, *in vivo* studies should be conducted. A transgenic mouse model of prostate carcinogenesis could be used to test the effects of obesity on the prostate tumor microenvironment. The prostate specific-PTEN knockout model will be a suitable option because it recapitulates disease progression similarly to how it is observed in humans. Another advantage to this model is that it is not as aggressive as other models and the effects of TAMs in disease progression could be evaluated, as well as macrophage infiltration to tumors at different stages of the disease. Studies in this model could be conducted to test whether CCL2 inhibition blocks macrophage recruitment to tumors. This could be done with CCL2 inhibitors that are currently in clinical trials for cancer therapy. To test whether TAM polarization is caused by PGE-2, non-steroidal inflammatory drugs (NSAIDs) that target the COX-2 pathway could be used. In addition, other mechanisms by which obesity may be promoting TAM polarization should be evaluated, because other molecules, aside of PGE-2, are also involved in this process. These molecules include, but are not limited to, IL-10, TGF-beta and M-CSF (Noy, 2014).

Future studies should also identify the circulating factors in sera from obese men that could be inducing prostate cancer cells to secrete macrophage recruiting and polarizing molecules. Conditioned media generated under obese conditions should be evaluated as well, with the same purpose.

In addition to *in vitro* and mouse models, human bio-specimens could be analyzed for presence of TAMs in tumor tissue. Tissue from tumor biopsies or from prostatectomies from obese and non-obese men could be used to assess whether obesity plays a role in macrophage recruitment. Additionally, an analysis looking at the association between BMI, tumor grade, Gleason scores and PSA levels with TAMs presence could be performed to see if there is a correlation between obesity, TAMs and cancer stage. However, it will be important to ensure that the appropriate markers for each of the macrophage subtypes are used. A recent study showed that TAM number was not associated with prostate-specific antigen (PSA) levels, Gleason score, or node status (Gollapudi, 2013). The authors of this study were identifying TAMs using a general tissue macrophage marker, CD68, rather than a more specific marker that better recognizes TAMs, such as CD206 or CD163.This could have led to a weak association between TAMs and advanced disease.

Given the need of a biomarker of progression in prostate cancer, and based on previous studies that show a connection between TAM presence and poor disease outcome, a study that investigates the potential of using TAM numbers as a predictive marker for aggressive prostate cancer is ongoing. As described in the appendix, this pilot study has

produced promising initial results. TAM infiltration was assessed in the tumor tissue in mice from a PTEN KO prostate cancer model, and was correlated with tumor-grade regardless of obesity status. This result coincides with clinical data indicating that TAM presence in tumors is correlated with advanced disease (Bingle, 2002).

To continue to determine the therapeutic potential of rapamycin in preventing prostate cancer progression by targeting TAMs, the role of mTOR in TAM survival should be explored further. For this assessment, a PTEN KO animal model would not be suitable, given that the PI3K/Akt/mTOR pathway will be mutated, so another transgenic mouse model of prostate cancer would have to be utilized. The focus was to look at ways to target TAMs in the prostate tumor microenvironment, however, if rapamycin is found to target TAMs selectively, this principle could be applied to any cancer with a high number of TAMs. Furthermore, the effects of rapamycin on the potential re-polarization of macrophages should be explored further. A study from Mercalli et al. has shown that rapamycin could not only reduce the number of M2 macrophages by inducing cell death in these cells, but also possibly by repolarizing them to an M1 subtype (Mercalli, 2013). Since the M1 subtype has been shown to be immune surveilling and tumor fighting, high M1 numbers could improve patient outcome. However, further research is needed in this area.

Now more than ever, we understand the potential of the immune system to slow cancer progression. Due to the large presence of immune cells in tumors, sometimes comprising more than 50% of total tumor mass (Mills et al., 2016), cancer researchers and immunologists have focused their attention on developing immunotherapies. The

innovation of these therapies comes from the idea of using the human body and its tools to combat cancer, in a more natural, less-toxic and less-invasive manner than the traditional treatments, such as chemotherapy and surgery. There is an increased recognition that immunity can be modulated to combat cancer. However, most of the immunotherapies are directed toward increasing T-cell infiltration and activation in tumors, and little attention has been paid to the presence of macrophages. TAM-focused therapies are an innovative approach that could be used alone or complementary to existing chemotherapies and immunotherapies. While macrophage-based immunotherapy is the latest breakthrough in immunotherapies, a better understanding of possible side effects, and how to maintain a balance of innate immunity, is still needed.

In conclusion, these studies have enhanced the understanding of the role that obesity plays in modulating TAMs presence in the tumor microenvironment, and the mechanisms by which it may promote prostate cancer progression. Hopefully, this knowledge will serve as basis for the development of future studies to achieve the goal of improving the outcome of prostate cancer patients. By further researching the connections between obesity and prostate cancer, it will be possible to identify clinically relevant targets that result in the development of effective therapeutics, and ultimately improve survival in obese prostate cancer patients.

Appendix A

TAM Concentration as a Biomarker of Prostate Cancer Aggressiveness

While prostate cancer remains a leading cause of cancer-related deaths among men in the US, most prostate cancers are indolent, and do not require aggressive intervention to improve overall survival. Currently, prostate cancer patients with low-grade tumors stay under active surveillance, while those with high-grade tumors usually get a prostatectomy. However, it is still difficult to decide the course of action of patients with medium-grade tumors that do not seem very aggressive according to their PSA levels and Gleason scores, which has resulted in the overtreatment of non-life-threatening prostate cancers, decreased quality of life, and increased medical care costs. This is mainly due to the lack of additional biomarkers that could predict tumor behavior. To date, PSA levels and Gleason scores alone have not been able to fully predict what the aggressiveness of the prostate tumors. Therefore, there is a significant need to identify a biomarker that would allow clinicians to make a more informed decision about what to recommend to patients with medium-grade PCa.

Preliminary findings suggest that TAMs are important drivers of prostate cancer progression; thus, it was hypothesized that presence of TAMs may serve as a potential biomarker to distinguish indolent from aggressive prostate cancer.

TAM concentrations were examined in tumors from mice from a PTEN KO mouse model of prostate cancer. To investigate if a difference in TAM concentrations between

different mouse samples could predict the occurrence of indolent and aggressive prostate cancer, H&E staining was employed to assess tumor composition and cancer stage, and immunohistochemistry was utilized to stain for macrophages and TAMs. Antibodies for F4/80 and CD206 were used to identify general macrophages and TAMs, respectively.

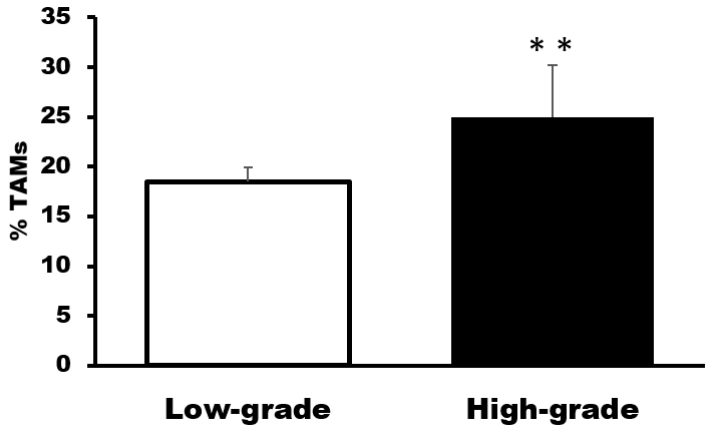

Figure A. Presence of TAMs is significantly more prominent in high-grade than low-grade tumors.

To assess TAM presence in tumors from mice from a prostate-specific PTEN KO model, tissues were stained with F4/80 and CD206 antibodies.

Results of this pilot study (**Figure A**) showed that high-grade tumors had higher concentrations of TAMs compared to low-grade tumors. Therefore, the number of TAMs in a tumor could be a promising biomarker for prostate cancer progression. Further investigation about the potential of TAM concentration as a marker for PCa progression is needed. Future studies should look at the presence of TAMs in biopsies of prostate cancer patients. This information should be correlated with Gleason score, cancer stage, PSA levels and clinical records of progression rates. TAM-specific markers should be used.

Bibliography

Allott EH, Hursting SD. Obesity and cancer: mechanistic insights from transdisciplinary studies. *Endocrine-related cancer*. 2015;22(6):R365-R386.

Allott EH, Masko EM, Freedland SJ. Obesity and Prostate Cancer: Weighing the Evidence. *European urology*. 2013;63(5):800-809.

Arendt, Lisa M et al. 2013. "Tumor and Stem Cell Biology Obesity Promotes Breast Cancer by CCL2-Mediated Macrophage Recruitment and Angiogenesis." *Cancer Res* 73(19): 6080–93.

Bingle, L., N. J. Brown, and C. E. Lewis. 2002. "The Role of Tumour-Associated Macrophages in Tumour Progression: Implications for New Anticancer Therapies." *The Journal of Pathology* 196(3): 254–65.

Blando, Jorge et al. 2011. "Dietary Energy Balance Modulates Prostate Cancer Progression in Hi-Myc Mice." *Cancer prevention research (Philadelphia, Pa.)* 4(12): 2002–14.

Bowers, Laura W. et al. 2014. "NSAID Use Reduces Breast Cancer Recurrence in Overweight and Obese Women: Role of Prostaglandin-Aromatase Interactions." *Cancer Research* 74(16): 4446–57.

Bowers, Laura W, and Linda A Degraffenried. "Targeting the COX-2 Pathway to Improve Therapeutic Response in the Obese Breast Cancer Patient Population." *Curr Pharmacol Rep*, 2015

Calle, Eugenia E, Carmen Rodriguez, Kimberly Walker-Thurmond, and Michael J Thun. 2003. “Overweight, Obesity, and Mortality from Cancer in a Prospectively Studied Cohort of U.S. Adults.” *n engl j med* 34817(17).

Cao, Yin, and Edward Giovannucci. 2016. “Obesity and Prostate Cancer.” : 137–53.

Casanovas, O., Hicklin, D. J., Bergers, G. & Hanahan, D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. 8, 299–309 (2005).

CDC, "Adult Obesity Facts | Overweight & Obesity | CDC.” 2016.

Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. Cancers. 2014;6:1670-1690.

Chen N, Zhou Q. The evolving Gleason grading system. C*hinese Journal of Cancer Research*. 2016;28(1):58-64.

Chiarugi P, Paoli P, Cirri P. Tumor microenvironment and metabolism in prostate cancer. Seminars in oncology. 2014;41:267-280.

Coffelt SB, Hughes R, Lewis CE. Tumor-associated macrophages: Effectors of angiogenesis and tumor progression. BBA - Reviews on Cancer. 2009;1796:11-18

Condeelis, J. & Pollard, J. W. Minireview Macrophages : Obligate Partners for Tumor Cell Migration , Invasion , and Metastasis. 263–266 (2006).

Cortez-Retamozo V, Etzrodt M, Newton A, et al. Origins of tumor-associated macrophages and neutrophils. Proceedings of the National Academy of Sciences of the United States of America. 2012;109:2491-2496.

De Beule N, De Veirman K, Maes K, et al. Tumour-associated macrophage-mediated survival of myeloma cells through STAT3 activation: TAM-mediated survival of myeloma cells. The Journal of Pathology. 2017;241:534-546.

Elchaki, Domenick J Falcone et al. 2014. "Macrophages JAK-Dependent IL-10 Expression in Metalloproteinase-9 Is Modulated by Mediated Induction of Matrix − IL-6 IL-6–Mediated Induction of Matrix Metalloproteinase-9 Is Modulated by JAK-Dependent IL-10 Expression in Macrophages." *The Journal of Immunology* 192: 0–0.

Epstein JI. An Update of the Gleason Grading System. *The Journal of Urology*. 2010;183(2):433-440.

Escamilla, J. *et al.* CSF1 Receptor Targeting in Prostate Cancer Reverses Macrophage-Mediated Resistance to Androgen Blockade Therapy. *Cancer Res* 75, 950–962 (2016).

Evans C, Glen A, Eaton C, et al. Prostate cancer proteomics: The urgent need for clinically validated biomarkers. *Proteomics Clinical Applications*. 2009;3:197-212.

Fan Q, Jing Y, Yu G, et al. Tumor-associated macrophages promote cancer stem cell-like properties via transforming growth factor-beta1-induced epithelial-mesenchymal transition in hepatocellular carcinoma. Cancer Letters. 2014;352:160-168.

Finley, David S. et al. 2009. "Periprostatic Adipose Tissue as a Modulator of Prostate Cancer Aggressiveness." *The Journal of Urology* 182(4): 1621–27.

Freedland, Stephen J. 2005. "Obesity and Prostate Cancer: A Growing Problem."

Clinical Cancer Research 11(19).

Fujimoto, Hiroshi et al. 2009. "Stromal MCP-1 in Mammary Tumors Induces Tumor-Associated Macrophage Infiltration and Contributes to Tumor Progression." *International Journal of Cancer* 125(6): 1276–84.

Giovannucci, Edward et al. 2007. "Risk Factors for Prostate Cancer Incidence and Progression in the Health Professionals Follow-up Study." *International journal of cancer* 121(7): 1571–78.

Gong, Z. et al. 2006. "Obesity, Diabetes, and Risk of Prostate Cancer: Results from the Prostate Cancer Prevention Trial." *Cancer Epidemiology Biomarkers & Prevention* 15(10): 1977–83.

Goswami, S. *et al.* Macrophages Promote the Invasion of Breast Carcinoma Cells via a Colony-Stimulating Factor-1 / Epidermal Growth Factor Paracrine Loop. 5278–5284 (2005).

Gregory CW, He B, Johnson RT, et al. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer research*. 2001;61:4315.

Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. *Cell*. 2010;140:883-899.

Hanahan D, Weinberg R. Hallmarks of Cancer: The Next Generation. Cell. 2011;144:646-674.

Hanahan, Douglas et al. 2012. "Accessories to the Crime: Functions of Cells Recruited to

the Tumor Microenvironment." *Cancer cell* 21(3): 309–22.

Heusinkveld, M. *et al.* M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. *J. Immunol.* 187, 1157–65 (2011).

Huang, J. et al. 2004. "Finding New Components of the Target of Rapamycin (TOR) Signaling Network through Chemical Genetics and Proteome Chips." *Proceedings of the National Academy of Sciences* 101(47): 16594–99.

Hursting, Stephen D., and Sarah M. Dunlap. 2012. "Obesity, Metabolic Dysregulation, and Cancer: A Growing Concern and an Inflammatory (and Microenvironmental) Issue." *Annals of the New York Academy of Sciences* 1271(1): 82–87.

Ito, Yusuke et al. 2015. "Adipocyte-Derived Monocyte Chemotactic Protein-1 (MCP-1) Promotes Prostate Cancer Progression through the Induction of MMP-2 Activity." *The Prostate* 75(10): 1009–19.

Iyengar, Neil M., Clifford A. Hudis, and Andrew J. Dannenberg. 2015. "Obesity and Cancer: Local and Systemic Mechanisms." *Annual Review of Medicine* 66(1): 297–309.

Jeong SK, Kim JS, Lee CG, et al. Tumor associated macrophages provide the survival resistance of tumor cells to hypoxic microenvironmental condition through IL-6 receptor-mediated signals. Immunobiology. 2017;222:55-65.

Jézéquel P, Campion L, Spyratos F, et al. Validation of tumor-associated macrophage ferritin light chain as a prognostic biomarker in node-negative breast cancer tumors:

A multicentric 2004 national PHRC study. *International Journal of Cancer.* 2011;131(2):426-437.

Jon G Quatromoni, Evgeniy Eruslanov. 2012. "Tumor-Associated Macrophages and Lung Cancer." *Am J Transl Res* 4(4): 376–89.

Kaler P, Galea V, Augenlicht L, Klampfer L. Tumor Associated Macrophages Protect Colon Cancer Cells from TRAIL-Induced Apoptosis through IL-1 beta-Dependent Stabilization of Snail in Tumor Cells. *PLOS ONE*. 2010;5:e11700.

Kattan MW. Re: Updated Nomogram to Predict Pathologic Stage of Prostate Cancer Given Prostate-Specific Antigen Level, Clinical Stage, and Biopsy Gleason Score (Partin Tables) Based on Cases from 2000 to 2005. *European Urology.* 2007;52(5):1528.

Kessenbrock, K., Plaks, V. & Werb, Z. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. Cell 141, 52–67 (2010).

Kim, S. W. *et al.* Consistent interactions between tumor cell IL-6 and macrophage TNF-a enhance the growth of human prostate cancer cells in the bone of nude mouse. *Int Immunopharmacol.* 11, 859–869 (2012).

Kremer, Celeste L. et al. 2006. "Expression of mTOR Signaling Pathway Markers in Prostate Cancer Progression." *The Prostate* 66(11): 1203–12.

Kurahara H, Shinchi H, Mataki Y, et al. Significance of M2-Polarized Tumor-Associated Macrophage in Pancreatic Cancer. Journal of Surgical Research. 2011;167:e211-e219.

Lanciotti M, Masieri L, Raspollini MR, et al. The role of M1 and M2 macrophages in prostate cancer in relation to extracapsular tumor extension and biochemical recurrence after radical prostatectomy. BioMed research international. 2014;2014:486798.

Levental, K. R. *et al.* Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell* 139, 891–906 (2009).

Lewis, Claire E., and Jeffrey W. Pollard. 2006. "Distinct Role of Macrophages in Different Tumor Microenvironments." *Cancer Research* 66(2): 605–12.

Li C, Shintani S, Terakado N, Nakashiro K, Hamakawa H. Infiltration of tumor-associated macrophages in human oral squamous cell carcinoma. Oncology Reports. 2002;9:1219-1223.

Lin, B. E. Y., Nguyen, A. V, Russell, R. G. & Pollard, J. W. Colony-stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy. 193, (2001)

Lissbrant, I F et al. 2000. "Tumor Associated Macrophages in Human Prostate Cancer: Relation to Clinicopathological Variables and Survival." *International journal of oncology* 17(3): 445–51.

Liu Y, Cao X. The origin and function of tumor-associated macrophages. Cellular & Molecular Immunology. 2015;12(1):1-4.

Loberg, R. D. *et al.* Targeting CCL2 with Systemic Delivery of Neutralizing Antibodies Induces Prostate Cancer Tumor Regression In vivo. 9417–9425 (2007).

Loeb S, Bjurlin M, Nicholson J, et al. Overdiagnosis and Overtreatment of Prostate Cancer. *European Urology*. 2014;65:1046-1055.

Luchman, H Artee et al. 2008. "The Pace of Prostatic Intraepithelial Neoplasia Development Is Determined by the Timing of Pten Tumor Suppressor Gene Excision." *PloS one* 3(12): e3940.

Mallat Z. Macrophages. Arteriosclerosis, Thrombosis, and Vascular Biology. 2014;34:2509-2519.

Mantovani, Alberto et al. 2002. "Macrophage Polarization: Tumor-Associated Macrophages as a Paradigm for Polarized M2 Mononuclear Phagocytes." *Trends in Immunology* 23(11): 549–55.

Mercalli, Alessia et al. 2013. "Rapamycin Unbalances the Polarization of Human Macrophages to M1." *Immunology* 140(2): 179–90.

Mills C. Anatomy of a discovery: M1 and M2 macrophages. Frontiers in Immunology. 2015;6:212.

Mills, C. D., L. L. Lenz, and R. A. Harris. 2016. "A Breakthrough: Macrophage-Directed Cancer Immunotherapy." *Cancer Research* 76(3): 513–16. Møller H, Roswall N, Van Hemelrijck M, et al. Prostate cancer incidence, clinical stage and survival in relation to obesity: A prospective cohort study in Denmark. *International Journal of Cancer*. 2015;136:1940-1947.

Mills, C D, J Shearer, R Evans, and M D Caldwell. 1992. "Macrophage Arginine Metabolism and the Inhibition or Stimulation of Cancer." *Journal of immunology*

(Baltimore, Md. : 1950) 149(8): 2709–14.

Mizutani, K. *et al.* The Chemokine CCL2 Increases Prostate Tumor Growth and Bone Metastasis through Macrophage. *Neoplasia* 11, 1235–1242 (2009).

Mukherji D, Omlin A, Pezaro C, Shamseddine A, de Bono J. Metastatic castration-resistant prostate cancer (CRPC): preclinical and clinical evidence for the sequential use of novel therapeutics. *Cancer and Metastasis Reviews*. 2014;33:555-566.

Nakanishi, Yuki et al. 2011. "COX-2 Inhibition Alters the Phenotype of Tumor-Associated Macrophages from M2 to M1 in ApcMin/+ Mouse Polyps." *Carcinogenesis* 32(9): 1333–39.

Nonomura N, Takayama H, Nakayama M, et al. Infiltration of tumour-associated macrophages in prostate biopsy specimens is predictive of disease progression after hormonal therapy for prostate cancer. *BJU International*. 2010;107(12):1918-1922.

Noy, Roy, and Jeffrey W. Pollard. 2014. "Tumor-Associated Macrophages: From Mechanisms to Therapy." *Immunity* 41(1): 49–61.

Obermajer, Natasa et al. 2011. "Positive Feedback between PGE2 and COX2 Redirects the Differentiation of Human Dendritic Cells toward Stable Myeloid-Derived Suppressor Cells." *Blood* 118(20): 5498–5505.

Ogden, C. L., Carroll, M. D., Kit, B. K. & Flegal, K. M. Prevalence of Childhood and Adult Obesity in the United States, 2012. doi:10.1001/jama.2014.732

Ohno S, Ohno Y, Suzuki N, et al. Correlation of Histological Localization of Tumor-associated Macrophages with Clinicopathological Features in Endometrial Cancer. Anticancer Research. 2004;24:3335-3342.

Olefsky, Jerrold M., and Christopher K. Glass. 2010. "Macrophages, Inflammation, and Insulin Resistance." *Annual Review of Physiology* 72(1): 219–46.

Olsson, A. *et al.* Tasquinimod triggers an early change in the polarization of tumor associated macrophages in the tumor microenvironment. *J. Immunother. Cancer* 1–14 (2015).

Onita T, Ji PG, Xuan JW, et al. Hypoxia-induced, Perinecrotic Expression of Endothelial Per–ARNT–Sim Domain Protein-1/Hypoxia-inducible Factor-2α Correlates with Tumor Progression, Vascularization, and Focal Macrophage Infiltration in Bladder Cancer. Clinical Cancer Research. 2002;8:471-480.

Peponi, Evangelia et al. 2015. "Inhibition of AKT/mTOR Signaling Induces Cell Cycle Arrest and Apoptosis in Mantle Cell Lymphoma." *Blood* 106(11).

Pollard, J. W. Tumor-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* 4, 1–8 (2004).

Rhee I. Diverse macrophages polarization in tumor microenvironment. Archives of Pharmacal Research. 2016;39:1588-1596.

Ruffell, Brian, Nesrine I Affara, and Lisa M Coussens. 2012. "Differential Macrophage Programming in the Tumor Microenvironment Macrophages in Solid Malignancies." *Trends Immunol* 33(3): 119–26.

Ruffell, Brian, and Lisa M. Coussens. 2015. “Macrophages and Therapeutic Resistance in Cancer.” *Cancer Cell* 27(4): 462–72.

Sartipy, Peter, David J Loskutoff, and Jeffrey M Friedman. “Monocyte Chemoattractant Protein 1 in Obesity and Insulin Resistance.”

Scheel, Christina et al. 2011. “Paracrine and Autocrine Signals Induce and Maintain Mesenchymal and Stem Cell States in the Breast.” *Cell* 145: 926–40.

Schmieder, Astrid et al. 2012. “Differentiation and Gene Expression Profile of Tumor-Associated Macrophages.” *Seminars in Cancer Biology* 22(4): 289–97.

Shapiro, A.M. James et al. 2000. “Islet Transplantation in Seven Patients with Type 1 Diabetes Mellitus Using a Glucocorticoid-Free Immunosuppressive Regimen.” *New England Journal of Medicine* 343(4): 230–38.

Shimura, Satoru et al. 2000. “Reduced Infiltration of Tumor-Associated Macrophages in Human Prostate Cancer: Association with Cancer Progression.” *Cancer Research* 60: 5857–61.

Sica, Antonio et al. 2008. “Macrophage Polarization in Tumour Progression.” *Seminars in Cancer Biology* 18(5): 349–55.

Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA: A Cancer Journal for Clinicians*. 2017;67:7-30.

Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene. 2010;29:4741-4751.

Snell, G I. et al. 2001. “Sirolimus(rapamycin) Allows Renal Recovery in Lung and Heart

Transplant Recipients with Chronic Renal Impairment." *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 20(2): 163–64.

Soave D, Miguel M, Tome F, de Menezes L, Nagib P, Celes A. The Fate of the Tumor in the Hands of Microenvironment: Role of TAMs and mTOR Pathway. Mediators of Inflammation. 2016;2016:1-7.

Spahn M, Boxler S, Joniau S, Moschini M, Tombal B, Karnes RJ. What is the Need for Prostatic Biomarkers in Prostate Cancer Management? *Current Urology Reports*. 2015;16:1-7

Suárez C, Morales-Barrera R, Ramos V, et al. Role of Immunotherapy in Castration-Resistant Prostate Cancer (CRPC). *BJU International*. 2014;113:367-375.

Takahashi, Kazuhiko et al. 2003. "Adiposity Elevates Plasma MCP-1 Levels Leading to the Increased CD11b-Positive Monocytes in Mice." *The Journal of biological chemistry* 278(47): 46654–60.

Takanami I, Takeuchi K, Kodaira S. Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: Association with angiogenesis and poor prognosis. Oncology, 1999;57:138-142.

Tania, Mousumi, Md. Asaduzzaman Khan, and Junjiang Fu. 2014. "Epithelial to Mesenchymal Transition Inducing Transcription Factors and Metastatic Cancer." *Tumor Biology* 35(8): 7335–42.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA: A Cancer Journal for Clinicians*. 2015;65:87-108.

Ueno, Takayuki et al. "Significance of Macrophage Chemoattractant Protein-1 in Macrophage Recruitment, Angiogenesis, and Survival in Human Breast Cancer."

Vidal, A C et al. 2017. "Obesity and Prostate Cancer-Specific Mortality after Radical Prostatectomy: Results from the Shared Equal Access Regional Cancer Hospital (SEARCH) Database." *Prostate Cancer and Prostatic Diseases* 20(1): 72–78.

Wan S, Zhao E, Kryczek I, et al. Tumor-Associated Macrophages Produce Interleukin 6 and Signal via STAT3 to Promote Expansion of Human Hepatocellular Carcinoma Stem Cells. Gastroenterology. 2014;147:1393-1404.

Wang, Dingzhi, and Raymond N. DuBois. 2010. "Eicosanoids and Cancer." *Nature Reviews Cancer* 10(3): 181–93.

Weichhart, Thomas, and Marcus D. Säemann. 2009. "The Multiple Facets of mTOR in Immunity." *Trends in Immunology* 30(5): 218–26.

Xie, Jianling, Xuemin Wang, and Christopher G Proud. 2016. "mTOR Inhibitors in Cancer Therapy." *F1000Research* 5.

Zeigler-Johnson C, Morales KH, Lal P, Feldman M. The Relationship between Obesity, Prostate Tumor Infiltrating Lymphocytes and Macrophages, and Biochemical Failure. Boissonnas A, ed. PLoS ONE. 2016;11(8):e0159109.

Zeisberger, S. M. *et al.* Clodronate-liposome-mediated depletion of tumour-associated macrophages : a new and highly effective antiangiogenic therapy approach. *Br. J. Cancer* 95, 272–281 (2006).

Zhou N, Zhang Y, Zhang X, et al. Exposure of Tumor-Associated Macrophages to Apoptotic MCF-7 Cells Promotes Breast Cancer Growth and Metastasis. International Journal of Molecular Sciences. 2015;16:11966-11982.