The Pennsylvania State University

The Graduate School

College of Agricultural Sciences

OBESITY-INDUCED IMMUNE MODULATION IN PANCREATIC CANCER

A Dissertation in

Immunology and Infectious Disease

by

Shawntawnee Collins

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

December 2015

The dissertation of Shawntawnee Collins was reviewed and approved* by the following:

Connie J. Rogers
Assistant Professor of Nutritional Sciences
Dissertation Advisor
Chair of Committee

K. Sandeep Prabhu
Professor, Department of Veterinary and Biomedical Sciences

Pamela A. Hankey-Giblin
Professor, Department of Veterinary and Biomedical Sciences

Andrea Mastro
Professor, Department of Biochemistry and Molecular Biology

Gordon Jensen
Professor and Head, Department of Nutritional Sciences

Margherita T. Cantorna
Head or Chair of Graduate Program

*Signatures are on file in the Graduate School

ABSTRACT

Pancreatic cancer is the fourth leading cause of cancer-related deaths (1), and it is associated with a poor survival rate due to late detection. Pancreatic cancer is characterized by immunosuppression. In late stage disease, the tumor microenvironment shifts from one favoring tumor recognition and clearance to one that promotes the recruitment of immunosuppressive cells such as regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) which can exacerbate tumor growth (2). Thus, a better understanding of the immunosuppressive factors in pancreatic cancer and the development of novel strategies to counteract them is critical in the treatment of pancreatic cancer.

The first aim of this project was to characterize tumor growth, inflammatory milieu, MDSC subset-specific accumulation and the assessment of MDSC subset functional differences in a murine model of pancreatic cancer. Female C57BL/6 mice were placed on an AIN-76 diet and $1x10^6$ Panc.02 tumor cells were injected subcutaneously in the lumbar region of the back. Tumor growth and survival were monitored for 30-60 days post tumor implantation. Plasma levels of inflammatory mediators and growth factors known to drive pancreatic carcinogenesis and MDSC were quantified in tumor free (TF) and tumor bearing mice at 60 days post tumor implantation. Total MDSCs and MDSC subset accumulation was also quantified in TF and Panc.02 tumor bearing mice at days 30, 40, 50 and 60 post tumor implantation. The contribution of MDSCs to pancreatic tumor development was assessed by treating mice with either anti-Gr-1^+ or anti-IgG isotype antibodies, or PBS vehicle. Lastly, the ability of MDSC

subsets to inhibit T cell proliferation, and the gene expression profile of each MDSC subset were quantified. We demonstrated increased plasma levels of inflammatory mediators GM-CSF, IL-6, TNF-α, MCP-1, MIP-1α, VEGF and TGF-β in Panc.02 animals in comparison to TF controls. MDSCs accumulated over time, with a preferential accumulation of the Gr-$1^{Hi}$$CD11b^{+}$ subset, which is also characterized as $CD11b^{+}$ $Ly6G^{+}Ly6C^{Lo}$. Antibody depletion of MDSCs reduced Panc.02 tumor burden in comparison to both isotype and PBS treated controls. Additionally, MDSCs subsets from tumor bearing animals inhibited T cell proliferation and produced TGF-β, IL-6 and IL-10. These findings indicated that MDSCs accumulate in pancreatic tumor bearing mice, and can inhibit T cell proliferation and enhance pancreatic tumor growth.

The second aim of this project was to evaluate the effect of obesity on the emergence of immunosuppressive factors at work in the Panc.02 model, in particular MDSCs. We hypothesized that obesity contributed to tumor growth by exacerbating the immunosuppressive environment, via the accumulation of MDSCs. To generate lean, control and obese mice, C57BL/6 mice were placed on a 30% kcal carbohydrate calorie restricted diet, a 10% kcal from fat diet, and a 60 kcal% from fat diet for 16 weeks, respectively prior to tumor implantation. Mice on each dietary intervention were injected subcutaneously with $1x10^{6}$ Panc.02 cells and continued on their respective diets for 60 days post tumor implantation. At sacrifice, tumor weight, splenic immune function, and MDSC accumulation and function were assessed. Obesity significantly enhanced pancreatic tumor growth and reduced survival in Panc.02 tumor bearing mice in comparison to

lean and control mice. Obese Panc.02 tumor bearing mice had a greater accumulation of MDSCs in the spleen, tumor draining lymph node (TDLN), and tumor in comparison to lean Panc.02 tumor bearing mice. Obesity in tumor bearing mice increased both MDSC subsets in spleen, but only increased the monocytic subset in the tumor in comparison to lean mice. We also demonstrated that MDSCs subsets from lean, control, and obese Panc.02 tumor bearing animals suppressed T cell proliferation to the same extent. These findings demonstrate that obesity increases the accumulation of MDSCs in pancreatic tumor bearing mice and may contribute to the immunosuppressive environment, thereby enhancing tumor growth.

Results from the current study contribute to our understanding about the role of MDSCs in pancreatic cancer and the role of obesity on MDSC accumulation and function. Understanding the long-term health consequences of obesity on tumor-associated immunosuppressive factors may provide critical insight into the prevention and treatment of obesity-related cancers in the future.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

MDSC: myeloid derived suppressor cell

APC: antigen presenting cell

DC: dendritic cell

NK: natural killer cell

IL-1α: interleukin-1 alpha

IL-1β: interleukin-1 beta

IL-1RA: interleukin-1 receptor antagonist

IL-3: interleukin-3

IL-6: interleukin-6

IL-8: interleukin-8

IL-10: interleukin-10

TNF-α: tumor necrosis factor

TGF-β: transforming growth factor

MCP-1: monocyte chemotactic activating protein

CRP: C - reactive protein

PDAC: pancreatic ductal adenocarcinoma

PanIN: pancreatic intraepithelial neoplastic

WT: wild type

APN: adiponectin

STAT1: signal transducer and activator of transcription, 1

STAT3: signal transducer and activator of transcription, 3

VEGF: vascular endothelial growth factor

GM-CSF: granulocyte macrophage colony stimulating factor

G-CSF: granulocyte colony stimulating factor

SCF: stem cell factor

CAF: cancer associated fibroblasts

ARG-1: arginase-1

iNOS: inducible nitric oxide

ROS: reactive oxygen species

TF: tumor free

ACKNOWLEDGEMENTS

I would like to thank my adviser Dr. Connie Rogers for giving me the opportunity to join her lab, and do my dissertation research. My graduate education would not have been possible without her help. I want to sincerely thank her for sharing her knowledge time and expertise.

I would like to thank my lab mates, especially Huicui Meng, Bill Turbitt, Adam, Black, Donna Sosnoski, and Shizhao Duan for their support, encouragement, and help in my graduate research. It would not have been the same without you all.

I would like to thank my committee members, Dr. Pamela Hankey-Giblin, Dr. Sandeep Prabhu, Dr. Andrea Mastro, and Dr. Gordon Jensen for the all of their help, support, and suggestions these past years.

I would also like to thank Dr. Avery August and Dr. Bettiyana Rajanna, I would not be in graduate school had it not been for them. Also for there continued support and encouragement throughout this process. I am also grateful for the ASU-PSU Brides program.

I would also like to thank my family, friends, and the Friendship family, Word of Faith family, Unity family for support throughout these years.

My deepest gratitude goes to my mother, father, sisters and nephew. To Walter R. Collins III, Shelia S. Collins, Shashawndra N. Collins, Shawndrella C.

Collins and Maximus Williams, thank you for your unending love, support, encouragement, and prayers. I could not have done it without you!!!

Last but not least, I would be remiss if I did not acknowledge my Faith. Thank You Jesus my Lord, my Savior, who has kept me, strengthened me, encouraged me, sustained me, and provided for me throughout every step of this process. Thank You Jesus!! Forever grateful!

CHAPTER 1

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related deaths, and has a five-year survival rate of 5% (1). Late diagnosis and lack of highly sensitive screening contribute to poor survival rates of patients with pancreatic cancer (3). The current treatments for pancreatic cancer include surgery, chemotherapy, radiation, and targeted therapies depending on stage/grade of cancer (1). The most effective chemotherapy includes gemcitabine, and more recently (FOLFIRINOX) a combination of 5-fluorouracil, leucovorin, oxaliplatin, and irinotecan (4). The poor survival rates and lethality of pancreatic cancer convey the need for improved therapies for this disease.

The role of the immune system in regulating tumor growth is described (5, 6) as the “immune surveillance hypothesis”. Due to the complexity of the immune system in both controlling and promoting tumor growth, the “immune surveillance hypothesis” was re-vamped and is now termed the “cancer immunoediting hypothesis”. Cancer immunoediting can be described in three phases: the elimination, equilibrium and escape phase. The first phase is called the elimination phase in which a number of immune cells including $CD4^+$ T cells, $CD8^+$ T cells and NK cells recognize and clear tumor cells. When the elimination phase fails, tumor cells enter into the second phase termed the equilibrium phase. In this phase, tumor cells undergo genetic instability and/or immune selection, which allow them to become resistant to recognition and clearance by the immune system. In the last phase, termed

the escape phase, tumor cells escape and proliferate with acquired immunosuppressive capabilities to avoid recognition and clearance by antitumor mechanisms. The transformed cells migrate to distant sites (metastasize) and proliferate undetected by antitumor immune mechanisms.

In pancreatic cancer, the adaptive immune system is active early with anti-tumorigenic activity via cytotoxic T cell activity and lysis of tumor cells. Yu, *et al.* shows a reduction in the number and function of $CD8^+$ T cells in pancreatic cancer patients compared to healthy controls (7). Previous studies have shown that the infiltration of $CD4^+$ and $CD8^+$ T cells into the tumor was positively correlated with survival after surgical treatment in patients with pancreatic adenocarcinoma (8). These results suggest a role for immune suppression in pancreatic cancer patients.

Conversely, the role of the immune system in promoting tumor growth has also been studied. Tumor cells create an immunosuppressive environment devoid of effector cells in order to avoid recognition and clearance of the immune system (9). Tumor-induced production of TGF-β and IL-10 can inhibit effector cells and induce the expansion of Tregs (9). The expansion of Tregs also results in suppression of antitumor responses (10). Tumors can also produce inflammatory mediators and growth factors, including IL-10, VEGF, GM-CSF, and IL-6, which promote accumulation of immunosuppressive MDSCs (11), which, in turn, alter anti-tumor immunity (12).

In mice, MDSCs are characterized by the expression of Gr-1 and CD11b (13) and comprise only 2-5% of the normal splenocyte population (12). In normal development, immature myeloid cells generated in the bone marrow travel to peripheral organs and differentiate into mature granulocytes, macrophages and dendritic cells (DCs) (14). However, in pathological conditions such as cancer, immature myeloid cells are halted in their differentiation (12), do not mature, and take on a suppressive phenotype. MDSC can suppress both the innate and adaptive immune response including NK cell cytotoxicity (12, 15, 16) and T cell effector functions (e.g. proliferation, IFN-γ production) (17). The activation of MDSC results in the up-regulation of immunosuppressive factors including arginase (ARG-1), inducible nitric oxide (iNOS) and reactive oxygen species (ROS) (18) which can induce $CD8^+$ T cell tolerance (17).

MDSCs expand significantly in tumor bearing animals and humans as a result of numerous tumor-derived factors, many which are associated with chronic inflammation. The pro-inflammatory cytokines IL-10, IL-1β, IL-6 (16, 19, 20), PGE_2 (21) and growth factors, VEGF, GM-CSF, G-CSF all induce MDSC accumulation (12, 16, 22, 23).

MDSCs have previously been identified in cancer patients, and the accumulation of MDSCs correlates significantly with disease stage and severity (24-26). Increases in MDSCs are observed in pancreatic cancer patients (27) with a concurrent reduction in $CD8^+$ T cells (28). The abnormal production of immunosuppressive factors, TGF-β and IL-10, in pancreatic tumor patients skews T cell cytokine production in favor of a TH2 phenotype (29). These studies

demonstrate that MDSCs are elevated in pancreatic cancer patients in comparison to healthy controls, and contribute to immune suppression observed in late stage pancreatic carcinogenesis. These data suggest that pancreatic cancer patients harbor an inflammatory environment that favors tumor growth and MDSC expansion.

Pre-clinical studies have also assessed MDSC accumulation and function in transgenic (30, 31), orthotopic (32) and subcutaneous models (33-38) of pancreatic cancer. In murine models of pancreatic cancer, a general consensus among studies is that MDSCs accumulate in the spleen, tumor, and blood in comparison to non tumor bearing controls (30, 37-41). MDSC that accumulate in preclinical models of pancreatic cancer have the capacity to suppress T cell function (30, 38, 39, 41) and contribute to pancreatic tumor growth (30, 38, 41). These data are in accordance with the clinical literature that demonstrates a role for MDSCs in the growth and progression of pancreatic cancer. Due to the immunosuppressive role of MDSCs in pancreatic cancer, understanding the mechanisms underlying the expansion and function of MDSCs is crucial for the success of future immunotherapies.

Current risk factors for pancreatic cancer include smoking, diabetes and obesity (26). Modifiable risk factors such as obesity are potential targets for reduction of the incidence of pancreatic cancer. Over 64% of the U.S. adult population is either overweight or obese (42). Obesity increases the risk pancreatic cancer (43, 44). However the mechanisms underlying the link between obesity and pancreatic cancer are not fully understood. Previous

studies demonstrate that obesity leads to a state of chronic inflammation (45). Obesity also impairs immune function (46). Clinical studies have examined the effect of obesity on immune function. Karlsson *et al.* reports that obese patients have a decreased number and function of antigen specific memory T cells (46). Obesity also reduces antigen specific $CD8^+$ T cell responses to influenza virus (47). Obesity similarly impairs the differentiation of monocytes into IL-4 induced alternative macrophages (48) in comparison to control patients. Obesity likewise reduces the number and function of NK cells (49) and gamma delta T cells (50) in comparison to control patients. However, the impact of obesity on the expansion and function of immunosuppressive MDSCs has not been investigated. Thus, a goal of this project was to evaluate the effect of obesity on pancreatic tumor growth and on the emergence of immunosuppressive MDSCs in a murine model of pancreatic cancer. Specifically, we hypothesize that obesity induces an increase in immunosuppressive factors, which enhances pancreatic tumor growth.

Results from this project will contribute to our understanding of immune regulation mediated by MDSCs in a subcutaneous murine model of pancreatic cancer. Additionally, an understanding of the long-term health consequences of obesity on tumor-associated immunosuppressive factors will provide critical insight into the prevention and treatment of many obesity-related cancers in the future.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Obesity continues to be one of the leading health issues in the US and abroad. The prevalence of obesity has risen significantly over the past several decades, and there is no indication that this trend is declining (51). More than one third of the US adult population is either overweight or obese (51). The most common measure of adiposity is body mass index (BMI), calculated by dividing body weight (kilograms, kg) by height (in meters squared, m^2). Obesity is defined by body mass index or BMI (BMI > 30.0 kg/m^2) and is associated with poor health outcomes including diabetes, heart disease and stroke (52).

2.3. Overview of Obesity and Inflammation

Inflammation is a physiological response to tissue injury, damage, and infection. Increases in blood flow, vascular permeability, leukocytes, and inflammatory mediators characterize this response. Inflammation is coordinated by inflammatory mediators (e.g. TGF-β, platelet derived factors, TNF-α, IL-1, nitric oxide, and prostaglandins), immune cells (e.g. neutrophils, monocytes, eosinophils, and mast cells) and chemokines (e.g. MCP-1, and MIP-1-α). Neutrophils play a role early in the inflammatory response. They are involved in chemotaxis, phagocytosis, and the production of proteases, reactive oxygen intermediates, and release inflammatory mediators. Monocytes/macrophages arrive next are also important early mediators of the inflammatory response. They also exhibit increased phagocytosis, increase the release of inflammatory mediators IL-1, IL-6 and TNF-α, MIP, and MCP-1 to resolve the tissue injury.

IL-1 is produced by a variety of cells including T cells and macrophages and has many functions. IL-1 is important in fever, prostaglandin synthesis, T cell activation, B-cell activation and function, the production of collagen and fibroblast proliferation (53). IL-10 is produced by T cells and B cells and can inhibit antigen specific T cell proliferation, NK function and TH1 cytokine production (54). TNF-α is produced primarily by macrophages and is important in pro-inflammatory responses as well. TNF promotes the inflammatory response by acting as a chemoattractant; it stimulates phagocytosis of macrophages, and is important in the inflammatory response for fever and release of acute phase proteins (55). Many immune cells including T cells and macrophages produce IL-6 and activate cells of adaptive immunity (56). IL-6 stimulates acute phase proteins including CRP, induces maturation of B cells, and is involved in activation and differentiation of T cells (56). MCP-1 and MIPs are chemokines produced by T cells, monocytes, macrophages, fibroblasts, and keratinocytes. MCP-1 can activate macrophages, and MCP-1 is important in basophil histamine release, and promotes TH2 immunity. MCP-1 also is important in the migration of monocytes from the blood stream into tissues where they become macrophages. MIP promotes TH1 immunity and is important in antiviral defenses (56). TGF-β is produced by T cells and monocytes and can inhibit the proliferation and activation of NK cells and T cells (56).

The aforementioned inflammatory mediators including (IL-1β, IL-10, TNF-α, L-6, MCP-1, and MIP-1α) are necessary for physiological response to injury or insult; however, failure to resolve inflammation had been linked to chronic

disease. Obesity also induces a state of low-grade chronic inflammation. Obesity-associated inflammation is linked to macrophage infiltration into adipose tissue. This infiltration of macrophages into the adipose tissue results in increases of inflammatory factors including IL-1β, IL-10, TNF-α, L-6, MCP-1, MIP-1α, and leptin (57) and a reduction in adiponectin (APN).

The adipokines, leptin and APN, can also modulate adaptive immunity and contribute to inflammation. Leptin induces a switch towards TH1 cell mediated response by increasing TNF secretion (57) and promoting proliferation of T cells. APN can suppress production of IFN-γ in macrophages via IL-10 production. APN stimulates the production of prostaglandin E_2 (PGE_2) in fibroblasts (58). The aforementioned studies suggest a relationship between inflammatory mediators and adipokines in regulating immune function. Therefore, it is possible that the communication between adipocytes and macrophages via adipokines can enhance and sustain inflammation.

Expansion of adipocytes and increased macrophage infiltration in adipose tissues are responsible for increased production of cytokines and chemokines observed in obesity (59). It has been reported from clinical studies that obesity significantly elevates serum levels of IL-5, IL-10, IL-12, IL-13, IFN-γ, TNF-α, MCP-1 (60), IL-6, CRP, and TNF-α (62). Consequently, chronic inflammation may contribute to the pathology of obesity associated diseases, including cancer (61). There is overwhelming evidence that inflammation can promote tumor development and growth (63). Individuals with inflammatory bowel disease (IBD) have a greater risk of developing colon cancer; *Helicobacter pylori* causes gastric

ulcers and increases risk of gastric cancer (62); hepatitis C infection increases risk of bladder and colon cancer; and chronic pancreatitis is a risk factor for the development of pancreatic cancer (63, 64). IL-6 and TNF-α in addition to coordinating the inflammatory response, can also promote tumor growth, and induce angiogenesis (65).

Obesity-induced inflammatory mediators produced via macrophage and the adipose tissue can also activate various transcription factors including STAT3 and nuclear factor κB (NFκB). Both transcription factors are important in controlling cell survival, growth, proliferation, invasion, angiogenesis and numerous other processes. Similarly, these inflammatory pathways can also induce the production of chemokines and cytokines that attract immune cells and sustain the inflammatory response. Using a colitis associated cancer model, inactivation of NFκB in cells of the myeloid origin reduce tumor growth and inhibit IL-6 secretion (66). Fujioka *et al.* show that inhibition of NFκB reduces tumor growth, metastasis and angiogenesis of pancreatic cancer in orthotopic pancreatic cancer model. The constitutive activation of NFκB contributes to tumor cell proliferation, survival, and inflammation in pancreatic cancer (67). A human pancreatic cancer cell line also constitutively expresses NFκB (68). These studies demonstrate a role for NFκB in promoting pancreatic carcinogenesis.

To further provide a role for inflammation in cancer, studies have also examined the long-term use of nonsteroidal inflammatory drugs (NSAIDs) in relation to cancer. NSAIDs can reduce colon cancer risk by 40% (69) and aspirin reduces incidence in breast cancer patients (70). The mechanism of action for

NSAIDs is inhibition of cyclooxygenases (COXs) (71) and the ability to induce apoptosis of cancer cells (72). The use of NSAIDs and aspirin reduce cancer risk, and thus confirms the role for inflammation in the carcinogenesis process.

2.4. Overview of Obesity and the Immune System

Numerous studies have explored the effect of obesity on immune function (73). Beck *et al.* show that obesity can alter the innate and adaptive immune response in both animal models and human subjects (46). In clinical studies, being overweight and obese impairs the immune response to influenza vaccination, in comparison to normal weight controls (47). Obese participants have lower antibody titers 12 months post vaccination (47) and greater susceptibility to viral infection (74). Additionally, obesity increases risk of infection, slows wound healing, and is responsible for decreases in T cell and NK cell function (75, 76). Natural killer T cells from obese individuals show a significant reduction in expression of TNF-related apoptosis inducing ligand (TRAIL), and phosphorylation of janus kinase 2 (JAK2) in comparison to normal weight controls (49). Obesity also reduces the number and function of gamma delta T cells in humans (50). These studies demonstrate that obesity impairs immune function in humans.

Additionally, numerous innate and adaptive immune impairments are also observed in preclinical models of obesity. Winer *et al.* show a pathogenic role for B cells from obese mice, via the promotion of insulin resistance and glucose

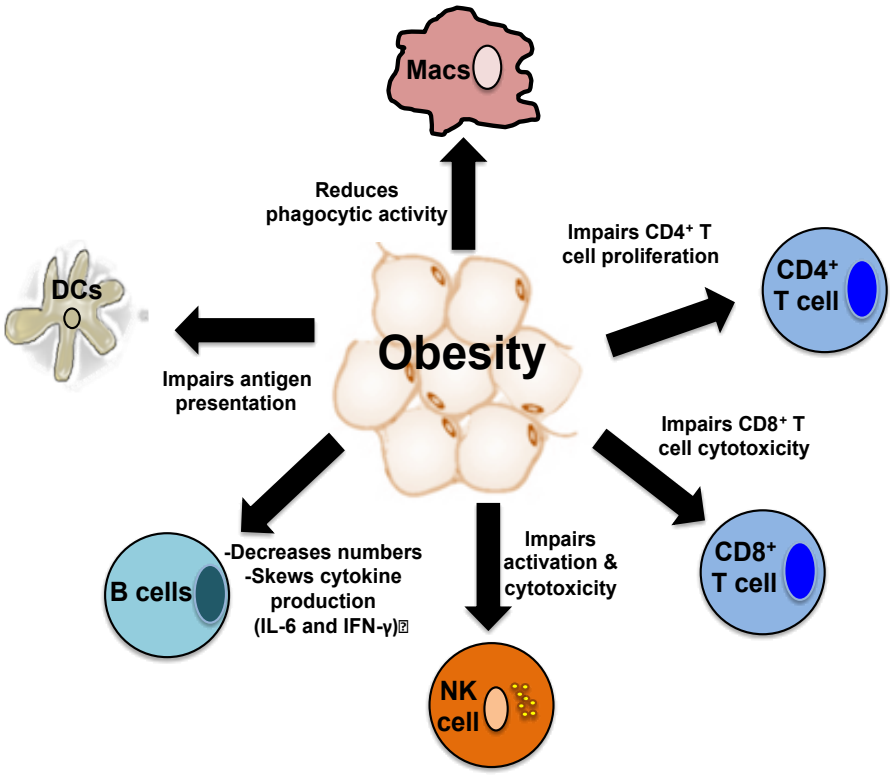

Figure 2.4. Obesity and the Immune System. Obesity can alter the innate and adaptive immune response in both pre-clinical and clinical models. Obesity decreases the number of B cells, and promotes inflammation via the production of pro-inflammatory cytokines (IFN-γ and IL-6). Obesity impairs the proliferation of $CD4^+$T and $CD8^+$T cells. Obesity impairs the cytotoxicity of $CD8^+$T cells. Obesity impairs antigen presentation of B cells. Obesity also impairs adaptive immunity. Obesity decreases the phagocytic activity of macrophages and increases the production of pro-inflammatory mediators (IL-6 and iNOS).

intolerance (77). DeFuria *et al.* also show that B cells produce pro-inflammatory cytokines (e.g. IL-6 and IFN-γ) and promote inflammation in obese mice in comparison to control animals (78). In addition to obesity-induced impairments in B cells, obesity also affects the function of T cells. Obesity increases infiltration and activation of $CD8^+$ T cell into the adipose tissue in comparison to control mice (79). A role for obesity in the accumulation of adipose tissue dendritic cells, which produce IL-6, IL-23 and TGF-β and generate Th17 cells (80) (which can

promote cancer) is also shown. In addition to its role on B cells and T cells, obesity also impairs antigen presentation of DCs (81), decreases the phagocytic activity of macrophages and increases the production of pro-inflammatory mediators (IL-6 and iNOS) in comparison to control mice (82).

Obesity also increases disease severity, and mortality to secondary influenza infection in mice, while impairing the generation and function of memory T cells (46). Previous studies in our lab demonstrate that the ability of antigen presenting cells (APCs) from obese C57BL/6 mice to induce T cell proliferation in comparison to lean animals is impaired (Rogers*, et al.* manuscript in preparation). Furthermore, antigen specific $CD4^+$T cell proliferation and $CD8^+$T cell cytotoxicity were also significantly decreased in obese animals. Thus, roles for obesity-induced impairments in immune function are well established.

The role of obesity on the emergence or function of immune suppressive cells is understudied. In a previous study by Xia *et al.*, immunosuppressive MDSCs accumulate in the bone marrow, blood, spleen, adipose tissue, and liver of obese (ob/ob) non-tumor bearing animals in comparison to wild type (WT) controls. MDSCs found in peripheral tissues improve insulin sensitivity, reduce serum IL-6 and TNF-α and skew macrophage polarization toward an M2 (pro-inflammatory) phenotype, suggesting that MDSCs may be negative regulators of obesity-induced inflammation in the absence of tumor (83). Wagner *et al*. reported that obesity deceases the number of circulating Tregs in comparison to normal weight controls (84). Decreases in Tregs were found in visceral adipose tissue from obese mice and obese patients (85). Zeyda *et al.* demonstrated

Tregs are more abundant in visceral adipose tissue in obese patients in comparison to lean control groups (86). Increases in MDSCs are in found the spleen and tumors of obese mice in an orthotopic renal cancer models in comparison to normal weight mice (87). These data demonstrate that obesity enhances immunosuppression via increasing the accumulation of immunosuppressive cells populations (e.g. Tregs and MDSCs).

2.5. Obesity and Pancreatic Cancer

Chronic inflammation contributes to the pathology of obesity-associated diseases including pancreatic carcinoma (63). Obesity is a modifiable risk factor for the development of several different types of solid cancers, including colon, breast and pancreatic cancer (88). Recent epidemiological studies suggest a positive association between obesity and pancreatic cancer risk (46, 81). A study by Arslan *et al.* used BMI as a measure of adiposity to determine the association between obesity and pancreatic utilizing the National Cancer Institute Pancreatic Cancer Cohort Consortium. In a pooled analysis of 14 cohort studies, BMI is associated with increased risk of pancreatic cancer (odds ratio =1.33, 95% CI, 1.12-1.58) when comparing BMI of <18.5 kg/m^2 to BMI of ≥ 35 kg/m^2. An association between BMI and pancreatic cancer risk is consistent among men and women (89). Jiao and colleagues also examined the association between obesity and pancreatic cancer using data from seven prospective cohort studies in collaboration with the National Cancer Institute including the National Institutes of Health-AARP Diet and Health Study (AARP), Agricultural Health Study (AHS), Breast Cancer Detection Demonstration Project (BCDDP), Prostate, Lung,

Colorectal and Ovarian Cancer Screening Trial (PLCO), US Radiologic Technologists Study (USRT); the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC); and the Shanghai Women's Health Study (SWHS). The results from this pooled analysis demonstrate the relative risk for a 5-kg/m^2 increment in BMI is in men is 1.06 (95% confidence interval (CI), 0.99–1.13) in men and 1.12 (95% CI, 1.05–1.19) in women (90). Renehan and colleagues utilized 221 data sets from Medline and Embase for all cancer sites, only using 16 specific data sets for pancreatic cancer. The populations were from North America, Europe, Australia, and Asia-Pacific. The relative risks for a 5 kg/m^2 increase in BMI for men and women in North America are RR=1.43 (95% CI, 1.19-1.72) and RR=1.16 (95% CI, 1.03-1.31). The relative risks for a 5 kg/m^2 increase in BMI for men and women in Europe and Australia are RR=1.08 (95% CI, 0.93-1.24) and RR=1.14 (1.05-1.23). The relative risks for a 5 kg/m^2 increase in BMI for men and women in Asia-Pacific are RR= 0.77 (0.54-1.11) and RR=1.34 (0.98-1.83). Population and gender differences are observed in the association between BMI and the incidence of pancreatic cancer, with the greatest increase in risk for pancreatic cancer in North American men in comparison to other populations (91). These data show gender differences among men and women, which could be potentially explained by different fat depots present in men in comparison to women. These results also suggest that BMI alone may not adequately capture the association between obesity and pancreatic cancer and other more sensitive measures of adiposity should be used. Most recently, a meta-analysis by Aune *et al.* examines the relationship

between obesity and pancreatic cancer risk utilizing BMI, waist to hip ratio and waist circumference as measures of adiposity. This meta-analysis includes 23 prospective studies examining the relationship between BMI and pancreatic cancer risk. The summary risk estimate (relative risk) for a 5-unit increase in BMI is RR=1.10 (95% CI, 1.07-1.14); for a 10-cm increase on waist circumference RR=1.11 (95% CI, 1.05-1.18) and for a 0.1 unit increase in waist to hip ratio was RR=1.19 (95% CI, 1.09-1.31) (43). Results from these epidemiologic studies support a positive association between obesity and pancreatic cancer.

Emerging evidence suggests that obesity may also influence pancreatic cancer mortality. A pooled analysis of 20 prospective studies from the National Cancer Institute BMI and Mortality Cohort Consortium demonstrates that a BMI of over 30 kg/m^2 increases mortality in pancreatic cancer patients (hazard ratio=1.43 (95% CI, 1.11-1.85) in comparison to a BMI of 21.0 to 23 kg/m^2. Increases in waist to hip ratio (0.1 unit increase, hazard ratio=1.09 (95% CI, 1.02-1.17) and waist circumference (10 cm increase, hazard ratio=1.07 (95% CI, 1.00-1.14) are also associated with increased mortality in pancreatic cancer patients. These results are similar for men and women. Another study also demonstrates a similar linear association between obesity and pancreatic cancer mortality, with a BMI >35 kg/m^2 conferring greater risk of mortality than a BMI between 30-34.9 kg/m^2 (92). Additionally, Kasenda *et al.* demonstrate that a 5 kg/m^2 increase in BMI is associated with increased mortality (hazard ratio =1.21 (95% CI, 1.06-1.41) in stage three and four pancreatic carcinoma patients. Lastly, Bethea *et al.* demonstrate a similar relationship between obesity and pancreatic cancer in

African Americans, using pooled data from seven prospective cohort studies (hazard ratio = 1.25 (0.99-1.57) for BMI 30-34.9 kg/m^2 and hazard ratio =1.35 (0.97-1.77) for BMI ≥35 kg/m^2) (93). These results, in total, demonstrate that obesity increases risk and reduces survival from pancreatic cancer patients.

2.6. Biological Mediators Underlying the Relationship between Energy Balance and Cancer Risk

The biological mechanisms underlying the link between obesity and cancer are not fully understood. Likely pathways linking obesity to pancreatic cancer are adipokines, inflammatory mediators (94) growth factors, and metabolic hormones (95).

Adipokines

In pathological states such as obesity, many metabolic mediators are dysregulated. Leptin, an adipokine involved with satiety is correlated with adipose tissue mass (73) and may play a role in obesity-related cancers. Increases in serum leptin levels are present in obese patients (85). Similarly, in animal studies, obesity also increases serum levels of leptin, while increasing tumor size and cellular proliferation in mice deficient for both leptin and its receptor (96, 97). Stolzenberg-Solomon *et al.* conducted a pooled analysis from three-cohort studies and demonstrate that high pre-diagnostic levels of leptin are associated with an increase in the risk of pancreatic cancer (odds ratio=2.55 (95% CI, 1.23-5.27). Furthermore, expression of leptin and its receptor (Ob-R) is related to colorectal tumor stage, metastasis and the signaling pathways mammalian target of rapamycin (m-TOR) phosphoinositide 3 kinase (PI3K), and serine/threonine

kinase (AKT) important in proliferation and apoptosis (98). Leptin also orchestrates vascular development and angiogenesis via VEGF in pre-neoplastic colon epithelial cells (99). Mendonsa *et al.* also demonstrate that leptin contributes to pancreatic tumor growth via PI3K/AKT *in vitro*. The role of leptin in pancreatic carcinogenesis is not fully understood; however, these data suggest that leptin can directly influence tumor growth by modulating proliferation, and vascularization of tumor cells in models of pancreatic cancer.

In addition to leptin, adiponectin (APN) may also play a role in the pathogenesis of cancer. Plasma levels of APN are inversely correlated with body mass index and visceral obesity (57). APN has potent anti-inflammatory effects in *in vitro studies*. Short-term treatment with APN on RAW 264.7 murine macrophage-like cells increases TNF-α and IL-10 production (100). Full length APN promotes a shift in the phenotype of M1 and M2 macrophages towards a more anti-inflammatory phenotype. In animal models of cancer, APN has an anti-proliferative effect on colon cancer cell lines (HCT116) (101). APN also inhibits NFκB activation in human aortic endothelial cells (102). NFκB is an important pathway, regulating survival, proliferation, inflammation, and immune regulation. Globular APN inhibits the proliferation of colon cancer cell through activation of AMPK and reduction in the mTOR pathway (103). These results suggest an anti-inflammatory role for APN on colon and pancreatic tumor cell lines. The relationship between APN and pancreatic cancer is less studied. In murine pancreatic cancer models, full-length APN inhibits proliferation of pancreatic cancer cells, and increases apoptosis and caspase activity in *vitro* (104). The

clinical data on examining the relationship between APN and pancreatic cancer is not clear. Serum levels of APN in pancreatic cancer patients were increasing, decreasing, or remain unchanged (105-107). The reduction of APNs anti-inflammatory effect observed in obesity may be responsible for enhanced tumor growth observed in obesity. Further research is warranted to understand the relationship between APN and pancreatic carcinogenesis.

Insulin & IGF

Insulin and insulin-like growth factors (IGF) are important in the regulation of glucose homeostasis and are often dysregulated in obesity. These factors are suggested as likely mediators of tumorigenesis. In preclinical models it was found that obesity increases tumor volume and levels of insulin in comparison to lean mice (96, 108). These animal studies provide a role for insulin in the pathology of pancreatic cancer. Hyperinsulinemia increases the risk for pancreatic cancer patients (109) by increasing levels of free IGF-1, which can enhance cell migration and pro-angiogenic factors potentiating tumor growth. Ben *et al.* conducted a meta-analysis examining the association between diabetes mellitus and risk of pancreatic cancer including 35 cohort studies. Ben *et al.* show that diabetes is associated with risk of pancreatic cancer (relative risk =1.94 (95% CI, 1.66-2.27). Previous studies show insulin resistance and IGF levels in the blood are associated with increased risk of colon cancer (110, 111). Insulin and IGF have previously been shown to activate signaling pathways AKT and PI3K. Insulin may enhance pancreatic cancer growth by modulation of signaling molecules AKT/PI3K, altering cell migration and enhancing tumor

growth. These pathways have pleiotropic effects, including cell growth and proliferation, which may be the mechanism by which they exert their effects in obesity-related cancers (95).

Inflammatory Mediators

Obesity is characterized by a state of low-grade chronic inflammation primarily driven by macrophage infiltration into the adipose tissue. Pro-inflammatory mediators thought play a role in obesity induced cancer risk includes: IL-1β, IFN-γ, IL-6, and TNF-α. Obesity significantly elevates serum levels of inflammatory mediators IL-10, IL-12, IL-13, IFN- γ, TNF-α and MCP-1 (60) in comparison to non-obese controls (112). Similarly, increases in serum levels of IL-6, CRP, and TNF-α are present in obese patients, and weight loss (via gastric surgery) significantly reduces serum levels of IL-6 and CRP (113). Pendyala and colleagues report that weight loss (via diet modifications) results in decreases in cytokines (e.g. TNF-α, and IL-8, IL-1β and MCP-1) and immune cell counts (e.g. T cells and macrophages) in mucosal biopsies. This study demonstrates that individuals who lost weight via diet manipulations had reduced colorectal inflammation (114). In cancer patients, Sullivan *et al.* found increases in levels of MCP-1 in obese pancreatic cancer patients in comparison to controls (60). Obesity increases serum levels of insulin, leptin, RANTES IL-1ra, and IFN-γ in a murine model of colon cancer (115). Park *et al.* demonstrate that obesity promotes carcinogenesis in a murine model of liver cancer, via TNF and IL-6, which induces chronic inflammation via STAT3 (116). The mechanisms linking obesity and cancer include increases in pro-inflammatory mediators TNF-α,

leptin, IL-1, IL-6, IL-8, resistin, MCP-1, and decreased anti-inflammatory, APN. These results suggest that obesity-induced inflammation may contribute to pancreatic carcinogenesis.

2.6. Overview of Pancreatic Cancer

Despite significant advances in research, cancer remains the second leading cause of death in the U.S. (1). In 2015, it is estimated that there will be over 1.6 million newly diagnosed cases of cancer and nearly 600,000 cancer-related deaths. Pancreatic cancer is the fourth leading cause of cancer deaths in the U.S. (1). Late diagnosis and lack of highly sensitive screening contribute to poor survival rates observed in patients with pancreatic cancer (3). The current five-year survival rate for pancreatic cancer patients is ~5% (1). At the time of detection, the disease is generally in an advanced stage and 75% of these patients die within one year of diagnosis (1). The current treatments for pancreatic cancer include surgery, chemotherapy, radiation, and targeted therapies depending on stage/grade of cancer (117). However, the side effects of the treatments including (e.g. nausea, vomiting, poor appetite, weight loss, diarrhea, hair loss, sores, increased chance of infection, and fatigue) and lethality of pancreatic cancer highlight the need for improved therapies for this disease. Thus, pancreatic cancer constitutes a significant public health threat, and is a cancer prevention and therapeutic challenge.

2.6.1. Pancreatic Ductal Adenocarcinoma (PDAC)

Pancreatic ductal adenocarcinoma (PDAC) is the predominant form of pancreatic cancer. PDAC is a stepwise progression from low-grade pancreatic intraepithelial neoplastic (PanIN) lesions to high-grade PanIN lesions, followed by PDAC (2). Genetic alterations are important components in the pancreatic carcinogenesis process. Kras, a GTPase responsible for cell cycling between the GTP-bound active and GDP-bound inactive state is the one the most commonly mutated genes in pancreatic cancer. 95% of pancreatic cancers have mutations in KRAS (118). Mutations of this oncogene lead to constitutive activation of KRAS, which leads to uncontrolled cell proliferation, anti-apoptosis, evasion of the immune response, and cell migration (119).

Mutations in P16/CDKN2A are also common in pancreatic cancer. P16 is an inhibitor of cyclin dependent kinase inhibitors (CDKIs), which inhibit CDK4/6 complexes causing them to become inactive resulting in cell-cycle arrest (118). The tumor suppressor gene, SMAD4, is a part of the TGF-β signaling pathway and is responsible for regulating the growth of epithelial cells. Inactivation in SMAD4 is observed in 45% of pancreatic cancers (120). P53 is another tumor suppressor gene frequently mutated in pancreatic cancer. P53 normally activates target genes in response to cellular stress by blocking the cell cycle transition, however in cancer mutations in this gene can lead to uncontrolled cell proliferation. Inactivation mutations in p53 occur in over 70% of pancreatic cancers (121). Pancreatic carcinogenesis is a multi-step process, with an accumulation of activating mutations in KRAS, and inactivating mutations in

p16/CDKN2A, p53, and SMAD4 genes, which are critical for the development of PDAC.

2.6.2. Cytokines and Chronic Inflammation in Pancreatic Cancer

Inflammation is a physiological response to injury, and or infection. In response to tissues injury, activation and migration of neutrophils and monocytes to the site of injury occur. Once at the site, monocytes/macrophages produce inflammatory mediators and chemokines including IL-6, IL-1 β, TNF- α, prostaglandins, CXCL8, CCL2, and CCL4 which stimulates other immune cell cells to the site of injury to clear response (56). The production of cytokines by macrophages also causes dilation of blood vessels, leading to movement of monocytes and neutrophils and leukocytes into the infected tissue (56). Collectively these changes cause the cardinal characteristic of inflammation including heat, pain, swelling and redness. Consequently, chronic inflammation contributes to the development of pancreatitis and pancreatic cancer (2) by increasing levels of pro-inflammatory mediators TNF-α, IL-6, IL-8, and IL-1β . Pancreatitis is a significant risk factor for the development of pancreatic cancer

Table 2.1. Inflammatory Mediators Important in Human Pancreatic Cancer

Cytokine	Function
IL-6	Positively correlates with tumor stage, cachexia and decreased survival
IL-8 (KC)	Associated with angiogenesis and metastasis negatively influences survival of tumor bearing (PC) mice associated with aggressiveness and invasiveness
IL-1α	IL-1α correlated with poor disease prognosis
IL-1β	IL-1β invasiveness, metastasis/migration
TNF-α	Association with cachexia, promotion of migration and metastasis and poor survival
MIF-1α	Enhancing proliferation, Induction of EMT, poor prognosis
MCP-1	Associated with pro-inflammatory macrophage infiltration
TGF-β	Promotion of immune evasion,and associated with poor prognosis

(122, 123). Elevations in levels of the inflammatory mediators TNF-α, IL-6, IL-8, and IL-1β play a role in pancreatic cancer. TNF-α is instrumental in invasion and metastasis (124). IL-6 can both promote angiogenesis and increase cell proliferation and is positively correlated with tumor stage and decreased survival in humans (125). IL-8 also promotes angiogenesis (126), migration and metastasis (127) in pancreatic cancer. Similarly, IL-1β increases migration, metastasis (128) and invasiveness of pancreatic cancer cells (129). An elevation in serum levels of TGF-β and IL-10 correlates with poor prognosis in cancer patients. Additionally, IL-10 in humans also correlates with tumor stage and promotes a pro-inflammatory shift in (29) patients with pancreatic cancer.

TGF-β is responsible for a variety of cellular processes including cell proliferation, embryogenesis and differentiation, angiogenesis, apoptosis, extracellular matrix remodeling and wound healing, and immunosuppression (130). However, contradicting roles for TGF-β exist in pancreatic cancer. TGF-β plays the roles of tumor suppressor and tumor promoter in pancreatic cancer. Overexpression of TGF-β in pancreatic cancer, via tumor-stromal interactions may lead to widespread immunosuppressive effects allowing tumor cells to evade anti-tumor immune responses, and thus promote pancreatic tumor growth. Serum levels of TGF-β rise in cervical cancer patients in comparison to control patients (131). Previous studies also demonstrate increases in serum levels of TGF-β in pancreatic cancer patients in comparison to healthy controls (132). Jacobs and colleagues associate serum levels of TGF-β with a higher risk of

pancreatic cancer (133). Future targeted therapies of TGF-β may provide promising options for pancreatic cancer treatment.

2.6.3. Cytokines and the Tumor Microenvironment

The tumor microenvironment is comprised of stromal cells, tumor cells, cancer-associated fibroblast, and immune cells. Numerous cells can secrete cytokines and growth factors within the tumor microenvironment. Stromal cells including cancer-associated fibroblasts (CAFs) produce TGF-β, IL-1, IL-6 and COX-2 (134). Similarly, tumor infiltrating immune cells (e.g. T cells, neutrophils, dendritic cells, macrophages, and mast cells) produce numerous growth factors (e.g. VEGF, GM-CSF, G-CSF) and cytokines (e.g. IL-1, IL-10, IL-10, TGF- β) that can also support tumor progression. Thus, the tumor microenvironment is important in the potentiation and development of pancreatic cancer.

2.6.4. Immune Suppression and Pancreatic Cancer

The role of immune suppression in pancreatic cancer progression is documented (2, 135, 136). Elevated levels of IL-10 and TGF-β are found in serum of pancreatic cancer patients (137). These immunosuppressive factors are also correlated with poor prognosis in cancer patients (138). Tumor cells utilize many different mechanisms to evade or suppress the host immune response. Antigen-specific T cell tolerance is one of the major mechanisms by which tumors escape immune cell destruction (139-141). Two major immunosuppressive cell populations that can promote tumor growth and suppress the host immune response are Tregs and MDSCs.

Yamamoto *et al*. show a significant increase in circulating Tregs in

pancreatic cancer patients in comparison to healthy controls (142). Similarly, Tang and colleagues show an increase in the accumulation of Tregs in pancreatic tumor tissue in comparison to normal pancreatic tumor tissues from patients without cancer. Additionally, this increase in Tregs in tumor tissue is related to a decrease in $CD8^+$T cells in the tumor (124). In a murine model of pancreatic cancer, the depletion of Tregs results in better survival of these animals (143), suggesting a role for immunosuppression by Tregs in the progression of disease. Increases in immunosuppressive Tregs are found in ductal adenocarcinomas, with a concurrent reduction in infiltration of intraepithelial $CD8^+$T cells in pancreatic ducts (144). These results demonstrate that Tregs contribute to immunosuppression in pancreatic cancer patients.

MDSCs suppress host immune responses (15). MDSCs also accumulate in pancreatic cancer patients, and their accumulation correlates with disease stage and severity (25, 26). Increases in MDSCs accumulation are present in pancreatic cancer patients with a concurrent reduction in $CD8^+$T cells (28). A recent study by Ghansah and colleagues establish a role for immune suppression in pancreatic cancer via MDSCs. They found that depletion of MDSCs in combination with a dendritic cell vaccination significantly delays tumor growth and improves survival in tumor bearing mice (145). Stromnes *et al.* show that depletion of granulocytic MDSCs in mice with pancreatic ductal adenocarcinoma increases $CD8^+$/IFN-γ^+ cells in the tumors of pancreatic tumor bearing mice $CD8^+$T cells and induces epithelial tumor cell apoptosis (30). Previous studies show that the infiltration of $CD4^+$ and $CD8^+$ T cells into the

tumor is positively correlated with survival after surgical treatment. These results demonstrate that in addition to Tregs, MDSCs also contribute to immune suppression in pancreatic cancer patients.

2.7.1 Phenotypic Characterization of Myeloid Derived Suppressor Cells

Murine MDSCs can be identified by expression of cell surface markers Gr-1 and CD11b. MDSCs can further be divided into two subtypes, granulocytic and monocytic MDSCs based on Gr-1 expression and/or additional markers Ly6G and Ly6C. Gr-1^{Hi}CD$11b^{+}$ ($CD11b^{+}Ly6G^{+}Ly6C^{Lo}$) cells are termed granulocytic MDSCs due to their granulocytic/neutrophil-like morphology (polymorphonuclear with dense granules). Gr-1^{Lo}CD$11b^{+}$ ($CD11b^{+}Ly6C^{Hi}Ly6G^{-}$) are termed monocytic MDSCs due to their monocyte-like morphology (mononuclear).

The expansions of these two MDSC subsets occur in cancer including colon, breast, and pancreatic cancer (146). MDSC subsets differ in their suppressive function in various experimental tumor models (12, 146). Inflammatory and tumor derived factors can activate MDSCs, which can result in increased production of immune suppressive agents such as arginase (ARG1), inducible nitric oxide (iNOS), and reactive oxygen species (ROS) (12). MDSC subsets mediate their suppressive function through several different mechanisms. Both MDSC subsets express immunosuppressive factors ARG-1 (12). Increased levels of ARG-1 in MDSCs can lead to the depletion of L-arginine, which can inhibit T cell proliferation (147, 148). MDSCs are also known

to produce TGF-β (149) and IL-10 (22) which both have the ability to suppress T cells responses including proliferation, activation, and differentiation (150, 151).

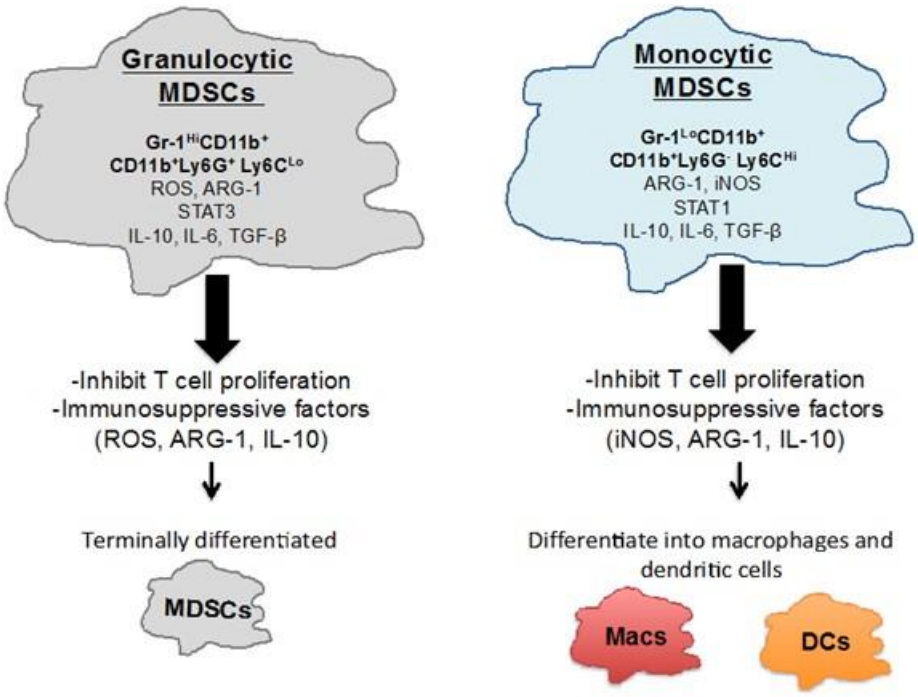

Figure 2.7.1. MDSC subset specific suppressive mechanisms. MDSCs subsets can inhibit T cell proliferation, and produce immunosuppressive factors. Granulocytic MDSCs can inhibit the proliferation of T cells via the production of ROS and ARG-1 through STAT3 mechanisms. Granulocytic MDSCs are terminally differentiated MDSCs. Monocytic MDSCs inhibit T cell proliferation of T cells via the production of iNOS and ARG-1 through STAT1 dependent mechanisms. Monocytic MDSCs can differentiate into macrophages (Macs) and dendritic cells (DCs). Both MDSC subsets produce immunosuppressive factors ARG-1 and IL-10.

2.7.2 Suppressive Capabilities of MDSCs

Adaptive Immunity

MDSCs are known to suppress immunity by altering both innate and adaptive host immune responses (17). The activation of these immature myeloid cells in disease states such as infection and cancer results in the upregulation of numerous immunosuppressive factors, e.g. ARG-1, iNOS, peroxynitrite, and ROS (12), which can result in the induction of antigen-specific $CD8^+$ T cell

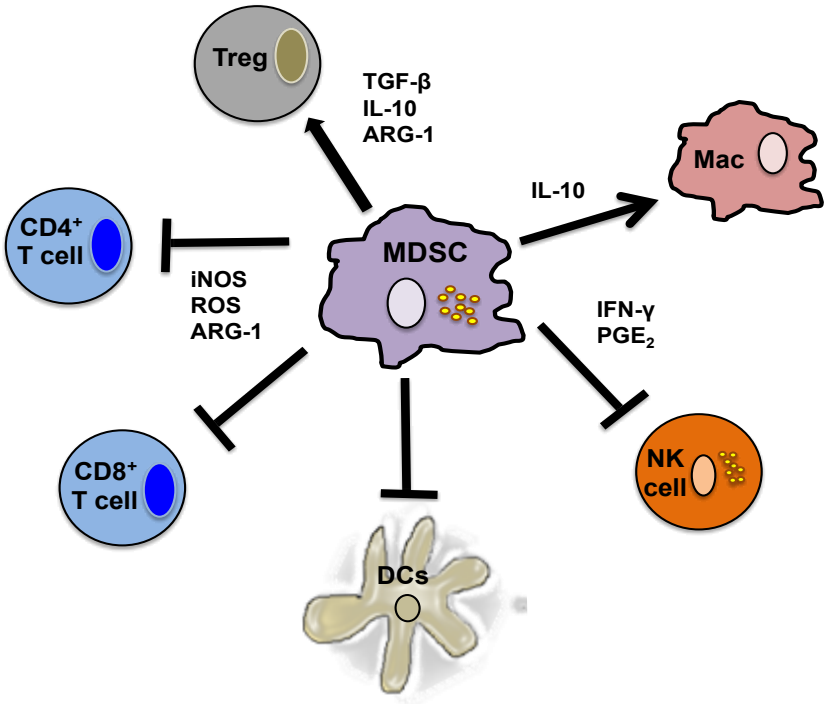

Figure 2.7.2. MDSC suppressive mechanisms. MDSCs inhibit both innate and adaptive immunity. MDSCs alter cytokine production of macrophages, polarizing them toward a tumor-promoting phenotype via the production of IL-10. MDSCs impair antigen presentation of DCs. MDSCs impair the function NK cells via cell contact dependent mechanisms including the production of PGE_2 decreases the expression of activating receptors (e.g. NKG2D, NKp46 and NKp44) on NK cells. MDSCs also inhibit the cytotoxicity of NK by inhibiting production of IFN-γ. MDSCs can drive the expansion of MDSC via the production of TGF-β, and IL-10. MDSCs suppress adaptive immunity by up-regulating ARG-1, iNOS, and ROS, which can result in the induction of antigen-specific T cell tolerance.

tolerance (17). Antigen-specific T cell tolerance is one of the major mechanisms by which tumors escape immune cell destruction (139-141).

MDSCs suppressive mechanisms include L-arginine depletion in the tumor microenvironment, depletion/deprivation of cysteine, and induction of Tregs. MDSCs can disrupt or downregulate the T cell receptor (TCR) ζ -chain molecules via peroxynitrite resulting in the inability of T cells to transmit signals for activation. MDSCs inhibit NK cell cytotoxicity (152-154). In addition to suppressing natural NK and T cell function, MDSCs prevent activation and proliferation of $CD4^+$ and $CD8^+$ T cells. The suppressive activity of MDSCs on T cells requires contact and can be antigen specific or nonspecific depending on the tumor environment, inflammatory mediators, and the localization of MDSCs and effector cells (155, 156). This interaction is dependent on MHC I expression. Similarly, MDSCs inhibit antigen specific $CD4^+$ T cell responses, and this was dependent on MHC II expression (157). These results demonstrate that MDSCs can inhibit adaptive immune responses in a non-specific and antigen specific manner.

Innate Immunity

MDSCs can alter function of macrophages, dendritic cells and NK cells. MDSCs can alter cytokine production of macrophages, polarizing them toward a tumor-promoting phenotype via the production of IL-10 (16). Similarly, MDSCs reduce the production of IL-12 from macrophages through IL-10 production (153). MDSCs can also alter antigen presentation of DCs (15). Up-regulation of calcium binding proteins (S100A8 and S100A9) in hematopoietic progenitor cells

impairs DC differentiation, while driving the accumulation of MDSCs (158). MDSCs can also impair the function NK cells via cell contact dependent mechanisms. Production of PGE_2 decreases the expression of activating receptors (e.g. NKG2D, NKp46 and NKp44) on NK cells (159). MDSCs also inhibit the cytotoxicity of NK cells by inhibiting perforin production (154) and by inhibiting production of IFN-γ (160). These data demonstrate that MDSCs inhibit innate immune responses.

MDSCs functional capacity is hypothesized to be subset specific. Transcription factors STAT1 and STAT3 can regulate MDSC expansion and contribute to the up-regulation of immunosuppressive factors. STAT1 is known for its role in increasing transcription of molecules related to apoptosis, angiogenesis and immune-surveillance while decreasing expression of molecules for growth inhibition. STAT3 is important in triggering cell survival and proliferation (161) and is documented as a major transcription factor regulating expansion of MDSCs (12). In a murine lymphoma tumor model, granulocytic MDSCs have higher levels of reactive oxygen species (ROS) (162) and lower levels of NO mediated through STAT3-dependent mechanism (12). In contrast, monocytic MDSCs have higher levels of iNOS (12) and lower levels of ROS mediated through STAT1 in a murine thyoma tumor model (163).

MDSCs expand significantly in tumor bearing animals and humans as a result of numerous tumor-derived factors, many of which are associated with chronic inflammation including IL-1β, IL-6, IL-10, TGF-β and PGE_2 (12, 15, 16, 19, 20). Additionally, two signaling molecules have been identified in the

accumulation of MDSCs, PLC-γ and β-catenin (164). Tumor cells and stromal cells can produce growth factors (e.g. GM-CSF, SCF, VEGF and MCF), which can also cause the expansion of MDSCs (11, 12, 23, 165). Similarly, MDSCs can also produce some of these factors that drive their expansion including TGF-β, IL-6, IL-10, and VEGF (166), generating a positive feedback loop. Growth factors VEGF and GM-CSF caused the expansion of MDSCs in melanoma, lymphoma murine cancer models (167). IL-1β and IL-6 are important in the accumulation of MDSCs in a murine mammary carcinoma model (16, 19). IL-6 aids in the recruitment of MDSCs with metastatic capacity in a murine breast cancer model (168). Lee and colleagues also demonstrate that levels of IL- 6 and MDSCs correlate with disease prognosis in squamous cell carcinoma patients. These studies reveal that MDSCs can expand in response to variety of growth factors and inflammatory mediators; however, this expansion of MDSCs is tumor model specific. In pancreatic cancer models little is known about factors that drive the expansion of MDSCs.

2.7.3 MDSCs in Cancer

MDSCs are characterized in a variety of cancers including, breast, colon, lung, renal, esophageal, gastric, melanoma, and prostate colon cancer (24). Similarly, MDSCs accumulate in pancreatic cancer patients and their accumulation correlates with disease stage (25, 26). Granulocytic MDSCs (Lin^- $HLA^-DR^-CD33^+CD11b^+CD15^+$) are significantly elevated in the blood of pancreatic cancer patients in comparison to healthy donors (169). In summary, MDSCs contribute to the immune suppression observed in pancreatic cancer

patients and targeted depletion of MDSCs or of the functionality of MDSCs may offer significant benefit to pancreatic cancer patients.

MDSCs also accumulate in preclinical models of pancreatic cancer including transgenic (30, 39), orthotopic (41) and subcutaneous models of pancreatic cancer (37, 38, 145). The suppressive capacity of MDSC in pancreatic tumor models is also under investigation. Porembka *et al.* and Ghanash *et al.* utilize a subcutaneous model (145, 170) and Stromnes *et al.* utilize a genetically engineered mouse model of pancreatic ductal adenocarcinoma (PDAC) (30). All of the aforementioned studies show that MDSCs accumulate in the tumor, spleen and bone marrow of tumor bearing mice. Additionally, MDSCs from tumor bearing mice suppress T cell proliferation. Increases in levels of VEGF are present as pancreatic tumors progress concurrently with the accumulation of MDSCs in the tumor, spleen, lymph node, and bone marrow (41). MDSCs isolated from the tumor express ARG-1 and iNOS and inhibit $CD8^+$ T cell proliferation. Kidiyoor *et al.* utilize a genetic model of PDA that also express the human MUC gene (KCM mice). MDSCs from KCM mice express immunosuppressive factors Cox-2 and IL-10, PGE_2, iNOS, and ARG-1, indicating that MDSCs from KCM mice have suppressive potential. Collectively, these data demonstrate that MDSCs accumulate in preclinical models of pancreatic cancer, and have the ability to suppress T cell function.

In addition to the characterization of MDSCs, and their suppressive capacity, the contribution of MDSCs on tumor growth has been studied in tumor

models. Stromnes *et al.* utilize a genetically engineered mouse model of PDAC and demonstrate that targeted depletion of the granulocytic MDSC subsets increases the accumulation of $CD8^+$ T cells at the tumor site and induces of apoptosis of tumor cells (171). Ghanash *et al.* also demonstrate that reduction of MDSCs via gemcitabine in combination with a dendritic cell vaccination induce immunity in the Panc.02 tumor model (145). Additionally, Vincent *et al.* show tumor-bearing mice (lymphoma) treated with 5-Fluorouracil (5FU) have a functional $CD8^+$ T cell response (172). Suzuki *et al.* reports that gemcitabine reduces the number of MDSCs in the spleen without altering the number of effector cells ($CD4^+$ and $CD8^+$ T cells) in lung and mesothelioma tumor models (153). 5FU reduces splenic and tumor MDSCs without altering the number of B cells, T cells, and DCs. Sildenafil treatment reduces MDSC accumulation at the tumor site and deceases serum levels of IL-6 and VEGF (41). IL-6 and VEGF are known factors that drive the expansion of MDSCs (12, 19). This consequent reduction of MDSCs in the tumor also restores activation of T cells in the tumor. These data provide a direct link between MDSCs and enhanced tumor burden, and contribute to the suppression of anti-tumor immune mechanisms in a murine pancreatic tumor model.

2.8. Rationale for Current Research

To date, much of the work examining MDSCs in cancer models has focused on identifying markers to define immunosuppressive cell populations and to understand how MDSCs inhibit the generation of antitumor immune responses. Additional studies are needed to better understand how obesity, an

important physiological and environmental factor, influences the production of immunosuppressive factors.

Obesity is associated with an increased risk and reduced survival from many forms of cancer. In fact, estimates from a large, prospective analysis on the obesity-cancer relationship suggest 14%-20% of all cancer deaths from a range of cancer types are attributable to overweight and obesity (14). The mechanisms underlying the link between obesity and cancer risk and/or progression are poorly understood (88, 173). Several energy balance-related host factors are known to influence tumor risk and progression, including adipokines, steroid hormones, growth factors, inflammatory mediators, and immune alterations. However, little is known about the effect of obesity on tumor-associated immune suppressive mechanisms. Thus, the goal of the current study was to determine the role of MDSCs in a murine model of pancreatic cancer.

The role of the immune system in pancreatic tumor growth and progression is well established, however the contribution of MDSCs on immune suppression observed in pancreatic cancer is not fully understood. Additionally, a goal of the current study was to determine if obesity alters the phenotype and function of MDSCs, and determine if an obesity-induced increase in MDSC is one mechanism underlying the obesity-induced increase in pancreatic tumor growth. Obesity is considered a pro-inflammatory state, with elevated cytokines originating from macrophage-infiltrated adipose tissue (174). **Therefore, we hypothesize that the pro-inflammatory environment of obesity triggers the accumulation of MDSC in obese mice.** It is possible that an increase in

immunosuppressive cells in obese animals may provide a robust inhibitory signal to prevent immune recognition of tumor antigens; which may be an important mechanism by which obesity enhances tumor development.

CHAPTER 3

Gr-1^{+}CD11b^{+} myeloid derived suppressor cells inhibit T cell proliferation and augment pancreatic tumor growth

3.1. Abstract

Pancreatic cancer is characterized by immune suppression and poor anti-tumor immunity. Immunosuppressive cells, including myeloid derived suppressor cells (MDSCs) are elevated in pancreatic cancer patients, and their accumulation correlates with disease progression and pancreatic cancer-related mortality. MDSCs accumulate in subcutaneous, orthotopic and transgenic models of pancreatic cancer. However, the role of MDSCs in pancreatic tumor progression, the accumulation of MDSC subsets in different lymphoid compartments, and the contribution of MDSCs to the immunosuppressive environment in pancreatic cancer models are unknown and understudied. Therefore, the goal of the current study was to evaluate the role of MDSCs in pancreatic tumor progression, and to characterize their accumulation and determine the functional characteristics of MDSC subsets in a subcutaneous (s.c.) pancreatic cancer model. C57BL/6 mice were injected with $1x10^6$ Panc.02 cells s.c. in the left lumbar region. Tumor growth and survival were monitored three times a week starting at day 13 post tumor implantation. Animals were followed from 30-60 days post tumor implantation for endpoint analysis or until animal reached ethical limit for tumor volume ($1.5cm^3$). Gr-1^+ MDSCs were depleted in a subset of mice to determine if a reduction in MDSC accumulation altered tumor progression. Increased plasma levels of inflammatory mediators GM-CSF, IL-6, TNF-α, MCP-1, MIP-1α, VEGF, and TGF-β were observed in Panc.02 animals in comparison to tumor free (TF) controls. MDSCs accumulated over time, with a preferential accumulation of the Gr-1^{Hi}CD$11b^+$ (CD$11b^+$Ly$6G^+$Ly$6C^{Lo}$) MDSC

subset. Antibody depletion of MDSCs significantly reduced Panc.02 tumor growth in comparison to PBS and isotype control antibody treated mice. Additionally, both MDSCs subsets from tumor bearing animals significantly inhibited T cell proliferation *in vitro* and produced TGF-β, lL-6 and IL-10. These data suggest that MDSC contribute to Panc.02 tumor growth.

3.2. Introduction

Pancreatic cancer is the fourth leading cause of cancer mortalities (1). Pancreatic cancer is also associated with immunosuppression, including elevated levels of inflammatory mediators such as IL-6 and TGF-β; the expansion of immunosuppressive cells including MDSCs; and an overall reduction of anti-tumor immunity (2, 175). Immunosuppressive Tregs are increased in ductal adenocarcinomas patients (31) with a concurrent reduction in infiltration of intraepithelial $CD8^+$ T cells in pancreatic ducts (144). Moreover, MDSCs have also been shown to accumulate in pancreatic cancer patients, and their accumulation correlates with disease severity (25).

MDSCs are known to alter both innate and adaptive immune responses by inhibiting the activation and proliferation of $CD4^+$ and $CD8^+$ T cells (15). The activation of MDSCs increases production of immune suppressive factors including ARG-1, iNOS, and ROS. MDSCs are currently being examined in several pre-clinical models in an attempt to better understand how to control and/or reverse their immunosuppressive contribution to pancreatic cancer progression. Current treatments for pancreatic cancer patients are ineffective and limited, and due to the strong immunosuppression and late detection observed in

pancreatic cancer patients, immunotherapeutic approaches targeting MDSCs may be viable strategies to increase survival. Several studies have examined the emergence of MDSCs using subcutaneous (37, 38), orthotopic (40, 41) and transgenic (30, 39) models of pancreatic cancer, and demonstrate a role for MDSC in pancreatic cancer progression. However, no studies to date have characterized the emergence of MDSC subsets and their functional characteristics.

The goal of the current study was to determine the role of MDSC in pancreatic tumor progression in a widely used subcutaneous model of pancreatic cancer; and to characterize MDSC subset accumulation and their functional properties. A better understanding of the role of MDSCs in late stage pancreatic carcinogenesis is important in understanding the biological mechanisms linking immune suppression in poor prognosis in pancreatic cancer patients.

3.3. Materials and Methods

3.3.1. Animals

Female 6-week-old C57BL/6 and BALB/c mice were obtained from Jackson Labs (Bar Harbor, MA). All mice were housed at the Pennsylvania State University in the Chandlee Laboratory animal facility; and maintained on a 12-hour light/dark cycle with free access to food and water. Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals." The Institutional Animal Care and Use Committee of the Pennsylvania State University approved all animal experiments.

3.3.2. Cell Culture

The murine ductal adenocarcinoma cell line Panc.02 were originally established through the induction of pancreatic tumors with 3-methylcholanthrene and serial s.c. transplantation in C57BL/6 mice (176). Panc.02 cells were obtained from Dr. Jeffrey Schlom (National Cancer Institute, National Institute of Health) and cultured in McCoy's 5A without G418 (Thermo Scientific; Logan, UT), and supplemented with 10% FBS (Gemini Bio Products; Sacramento, CA), 0.1mM non-essential amino acids (Mediatech; Manassas, VA), 1mM sodium pyruvate (Mediatech), 2mM glutamine (Mediatech), 10mM HEPES (Mediatech), 100U/mL penicillin streptomycin (Mediatech). Panc.02 Cells were maintained at 37°C and 5% CO_2 and passaged every 3-4 days using trypsin/EDTA (Mediatech).

3.3.3. Tumor Protocol

$1x10^6$ Panc.02 cells were injected in PBS subcutaneously (s.c.) into the lumbar region of C57BL/6 mice. Tumor growth was monitored three times per week with a digital caliper from day 13 post tumor implantation until endpoint analysis (day 30-60 post tumor implantation depending on the experiment) or until all mice reached the ethical limit for tumor size (1.5 cm^3). Tumor volume was calculated by multiplying the short side ^{x} short side ^{x} long side /2 ^{x}0.001 to get tumor volume in cm^3. For survival studies, mice were sacrificed when tumor size reached the ethical limit for tumor size of 1.5cm^3, and were counted as death for time to event analysis (survival analysis).

3.3.4. Immunological Assays

Isolation of immune cells

Spleens were harvested via gross dissection, and single cell suspensions of splenocytes were prepared from individual mice by mechanical dispersion, as previously described (177). Briefly, harvested spleens were mechanically disrupted with a syringe plunger and passed through a 70 µm nylon mesh strainer (BD Biosciences; Bedford, MA), erythrocytes were lysed with ACK lysing buffer (Lonza), washed twice in complete media RPMI 1640 (Mediatech), and supplemented with 10% FBS (Gemini Bio Products), 0.1mM non-essential amino acids (Mediatech), 1mM sodium pyruvate (Mediatech), 2mM glutamine (Mediatech), 10mM HEPES (Mediatech), 100U/mL penicillin streptomycin (Mediatech) and cell counts and viability were determined via trypan blue exclusion.

MDSC Cell Isolation

MDSCs were isolated from a single cell suspension of splenocytes as per manufacturer's instructions (MSDC Isolation kit; Miltenyi Biotec; Gladbach, Germany). Cell count was determined and up to $1x10^8$ splenocytes were centrifuged for 10 minutes at 300g at 4°C. Supernatants were aspirated and cells were re-suspended in 350µL of isolation buffer (PBS, 0.5% BSA, and 2mM EDTA). FcR blocking reagent (50µL) was added, vortexed, and incubated for 10 minutes in the refrigerator 4°C. After incubation 100µL of anti-Ly-6G-antibody was added to the cells, mixed well, and incubated for 10 minutes at 4°C. Cells were washed by adding 10mL of buffer and centrifuging at 300g for 10 minutes at 4°C. The cells were re-suspended in 800µL of buffer, then 200µL of anti-biotin microbeads were added, mixed well, and incubated for 15 minutes at 4°C. Cells

were washed by adding 10-20mL of buffer and centrifuging at 300xg for 10 minutes at 4°C. Aspirated supernatant off completely and the cell pellets were then resuspended in 500µL of buffer.

For magnetic separation with columns, LS columns were rinsed with 3mL of buffer and cell suspensions were added onto the column. Columns were washed three times with 3mL of buffer and the unlabeled cells that pass through were collected. The flow-through containing unlabeled cells was collected and placed on ice and labeled as theGr-1 ^{dim} Ly-6G^{-} cell fraction. The cells remaining on the column were collected by removing the columns from the magnet and flushing the columns with buffer. The collected cells were labeled as the Gr-1^{high} Ly-6G^{+} Granulocytic MDSC cells.

For further Isolation of the Gr-1^{dim} Ly-6G^{-}cells, the remaining cell suspensions were centrifuged at 300xg for 10 minutes at 4°C. The pellet was re-suspended in 400µL of buffer and 100µL of anti-Gr-1-antibody was added, mixed well, and incubated for 10 minutes at 4°C. Cells were washed by adding 5-10mL of buffer centrifuging at 300xg for 10 minutes at 4°C and the supernatant were aspirated. The cells were re-suspended in 900µL of buffer and 100µL of streptavidin microbeads were added, mixed well, and incubated for 15 minutes at 4°C. Cells were washed by adding 10-20mL of buffer and centrifuging at 300xg for 10 minutes at 4°C and the supernatants were aspirated completely. The cells were re-suspended in 500µL of buffer and magnetic separation was performed.

The MS column was placed in the magnetic field of a suitable MACS Separator and the column was prepared by rinsing with 500µL of buffer. The cell

suspension was applied onto the column. The unlabeled cells passed through the column. The column was rinsed 3 times with 500µL of buffer. The columns were removed from the magnet and flushed with buffer to collect the Gr-1 ^{dim}Ly-6G^{-} Monocytic MDSC fraction. After isolation MDSC subsets were counted and viability determined via trypan blue exclusion.

T cell and APC Isolation

T cells were isolated via negative selection using the Dynabeads® Untouched^{TM}Mouse T cells Kit (Life Technologies). APCs were isolated using the Dynabeads® Mouse Pan T (Thy1.2) Kit (Life Technologies). After isolation, T cells and APCs were counted and viability determined via trypan blue exclusion.

Flow cytometric analyses

Single cell suspensions of splenocytes were washed twice in PBS at 4°C. Cells were incubated with Fc block (Biolegend) and 1x10^6 cells were stained with saturating concentrations of conjugated antibodies including Gr-1 FITC (BD Biosciences; Bedford, MA), CD11b PE (BD Biosciences), Ly-6G APC (Biolegend) and Ly-6C APC-Cy7 (eBioscience; San Diego, CA) for 30 min at 4° C, as previously described (177). Following incubation with the conjugated antibodies, cells were washed twice in PBS and fixed in 1% paraformaldehyde. Lymphoid and myeloid cells were gated on forward vs. side scatter for a total of 30,000 events. All flow cytometric analyses were performed on a Beckman Coulter FC500 flow cytometer (Beckman Coulter; Indianapolis, IN). Flow cytometric analyses were plotted and analyzed using Flow Jo software (Tree Star; Ashland, OR).

Mixed Lymphocyte Reaction (MLR)

Isolated splenic T cells ($1x10^5$) from BALB/c animals were co-cultured with irradiated (2000 rads) APCs ($5x10^5$) isolated from Panc.02 tumor-bearing C57BL/6 mice and either ($Gr\text{-}1^{Hi}CD11b^+$ or $Gr\text{-}1^{Lo}CD11b^+$ MDSCs ($5x10^4$). Cells were incubated in flat-bottomed, 96-well plates and were pulsed with 1μCi per well of tritiated thymidine (H^3) at 72 hours. Proliferation was assessed by tritiated thymidine incorporation at 96 hours (Perkin Elmer; Waltham, MA) on a microbeta plate reader (Perkin Elmer; Waltham, MA). Each assay was performed in triplicate.

Cytokine Measurements

Plasma G-CSF, GM-CSF, IL-6, TNF-α, MCP-1, MIP-1α, and VEGF, and TGF-β, IL-6 and IL-10 from MLR culture supernatants were measured using the MILLIPLEX MAP Cytokine kit (Millipore; Billerica, MA). Mean fluorescence intensity was calculated from duplicate samples using the luminex-200 system and quantified on the Bio-Plex™ 200 System (Bio-Rad; Hercules, and CA). TGF-β was assessed in plasma and cell culture supernatants via ELISA (Biolegend) and quantified on an Epoch Microplate Spectrophotometer (Biotek; Winooski, VT). Each assay was performed in duplicate.

Gene Expression

Gene expression of iNOS, ARG-1, TGF-β, STAT1, STAT3, Cox-2, CD3, IL-10, and IL-6 was assessed in MDSC subsets from TF (n=4-6) and Panc.02-tumor bearing mice (n=3-5), and in cultured Panc.02 cells and *ex vivo* tumors from Panc.02 tumor bearing mice. Total RNA was extracted and genomic DNA

contamination was removed by using RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was quantified by using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), and reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies). Real-time qPCR was performed by using TaqMan real time PCR reagents (Life Technologies and an Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies). Primer sequences were based on published studies and actual primer sequences were listed in Supporting Information Table 1. Real-time qPCR data were calculated using the standard curve method and normalized to 18S RNA. Relative quantification or fold change in gene expression was determined using the $2^{\Delta\Delta}$Ct method using data from TF mice as reference control (178).

MDSC Depletion

MDSCs were depleted using an anti-Gr-1 mAb (clone RB6-8C5) (179). A rat IgG2b (clone LTF-2) mAb was used as isotype control to the Gr-1 mAb. Panc.02 tumor-bearing mice were injected with 100 mg of anti-Gr-1 mAb, isotype control mAb or PBS intraperitoneally (i.p.) starting at day 16 post tumor implantation, and every three days thereafter until day 40 post tumor implantation.

3.3.5. Statistical Analyses

All data are presented as the mean plus or minus the standard error mean. Differences in cytokine production were determined using two-tailed, unpaired *t*-tests assuming unequal variances. All cytokine data was skewed, thus the data were transformed (log transformation) prior to statistical analysis. Differences in cytokine production were assessed between groups via a Student

t-test or the Mann-Whitney test, depending on normality and variance. MDSC accumulation, MDSC-induced T cell suppression, and IL-10 production from MDSC subsets were assessed using one-way ANOVA, followed by Dunn's or Bonferroni correction for multiple comparisons where appropriate. Tumor growth following MDSC depletion and the number of MDSC subsets in TF and tumor-bearing mice were assessed using two-way ANOVA. For the gene expression data a fold change of greater than three was considered statistically significant. All analyses were conducted using GraphPad Prism 5 software (GraphPad Software; La Jolla, CA) and statistical significance was accepted at the $p \leq 0.05$ level.

3.4. Results

3.4.1. Tumor growth and survival characteristics.

Panc.02 tumor bearing mice (n=17) had palpable tumors by 19-day post implantation (Fig. 3.1a). By day 60-post tumor implantation, the mean tumor volume in Panc.02 tumor-bearing mice was 0.87 ± 0.31 cm^3 (Fig. 3.1a). Median survival for Panc.02 tumor-bearing animals (n=8) was 63 days post tumor implantation (Fig. 3.1b).

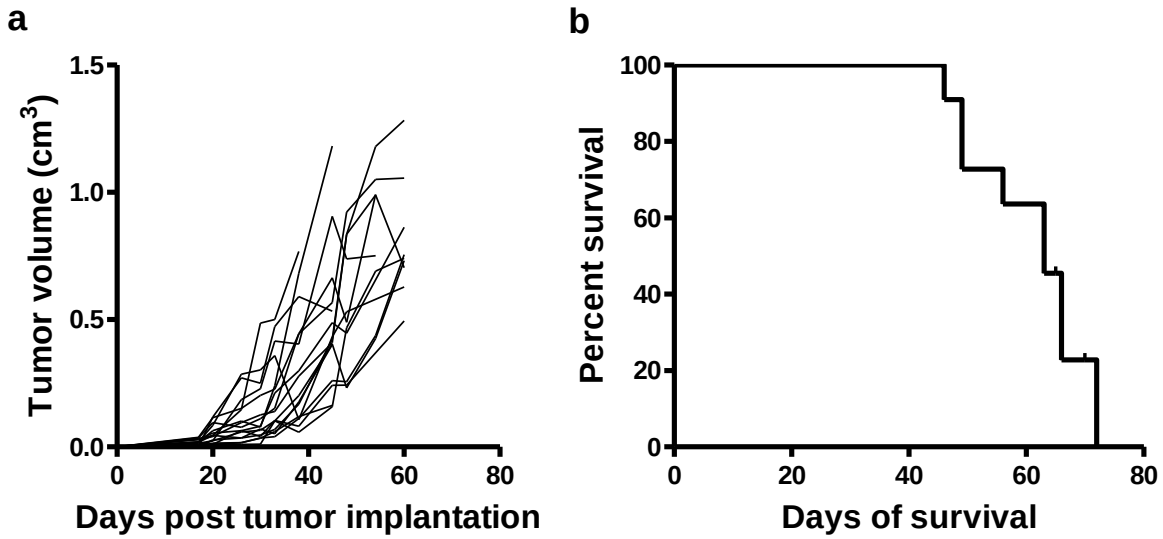

Fig. 3.1. Tumor growth and survival of Panc.02 tumor bearing animals. C57BL/6 animals were implanted s.c. with ($1x10^6$) pancreatic tumor cells (Panc.02) on day 0. Tumor growth was monitored three times per week with a digital caliper until 60 days or until all mice reached the ethical limit for tumor size (1.5 cm^3). Tumor growth over time in individual Panc.02 tumor-bearing animals (a, n=17). Median survival of Panc.02 tumor-bearing animals was 63 days (b, n=8)

3.4.2. Panc.02 tumor bearing animals have higher plasma levels of inflammatory mediators.

Cytokines were quantified in the plasma of TF and Panc.02 tumor (n=4-12/group) bearing mice at day 60-post tumor implantation. Panc.02 tumor-bearing mice had elevated levels of G-CSF (Fig. 3.2a, p=0.185), and significantly higher levels of GM-CSF (Fig. 3.2b, p=0.007), IL-6 (Fig. 3.2c, p=0.001), TNF- α (Fig. 3.2d, p<0.001) MCP-1 (Fig. 3.2e, p=0.001), MIP-1α (Fig. 3.2f, p=0.001), VEGF (Fig. 3.2g, p=0.042), and TGF-β (Fig. 3.2h, p=0.004) than TF animals. Additionally, plasma levels of TGF-β were significantly correlated with tumor weight in Panc.02 animals (data not shown; Pearson correlation r=0.699, p ≤0.050; and plasma levels of MCP-1 were significantly correlated with and tumor

volume in Panc.02 tumor bearing animals (data not shown; Pearson correlation r=0.617, p≤0.050).

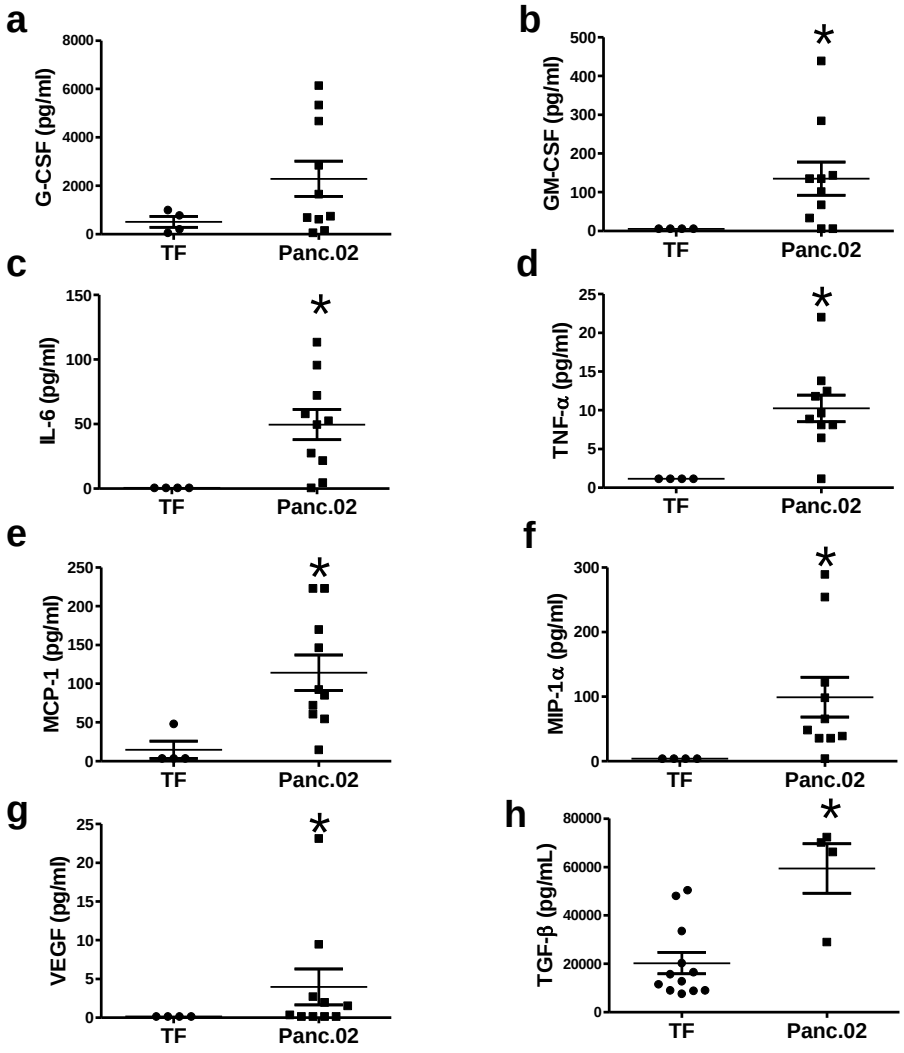

Fig. 3.2. Inflammatory mediators and growth factors in the plasma of TF and Panc.02 tumor bearing mice. Plasma levels of G-CSF (a), GM-CSF (b), IL-6 (c), TNF- α (d), MCP-1 (e), MIP-1α (f), and VEGF (g) were measured in TF (n=4/group) and Panc.02 (n=10/group) animals using the MILLIPLEX MAP Cytokine array at day 60 post tumor implantation. Plasma levels of TGF- β (h) were measured in TF (n=12/group) and Panc.02 (n=5-9/group) animals via

ELISA at day 60-post tumor implantation. A trend towards higher levels of G-CSF was observed in Panc.02 tumor-bearing animals in comparison to TF controls, however this did not reach statistical significance (a, t-test, p=0.185). Panc.02 tumor bearing animals had significantly higher levels of GM-CSF (b, t-test, p= 0.007), IL-6 (c, t-test, p=0.001), TNF- α (d, t-test, p< 0.001), MCP-1 (e, t-test, p= 0.001), MIP-1α (f, t-test, p=0.001), VEGF (g, Mann-Whitney, p=0.042), and TGF-β (h, t-test; p=0.004) in comparison to TF animals. Asterisk indicates a significant difference from TF animals.

3.4.3. Accumulation of Gr-1$^+$CD11b$^+$ cells in tumor bearing animals.

Splenocytes were isolated from TF (n=8/group) and Panc.02 tumor bearing animals at 30 (n=2/group), 40 (n=7/group), 50 (n=9/group) and 60 (n=5/group) days post tumor implantation to characterize the time course and magnitude of the tumor-associated increase in the Gr-1$^+$CD11b$^+$ population. The percentage of Gr-1$^+$CD11b$^+$ MDSCs was significantly greater at day 50 and 60 post tumor implantation as compared to TF mice (Fig. 3.3b, κ=14.90, day 50 p≤0.050 and day 60 p≤0.001). A similar trend was observed in the number of Gr-1$^+$CD11b$^+$ MDSCs that accumulated at day 50 and 60 post tumor implantation (Fig. 3.3b, κ=15.73, Dunn's multiple comparison test: day 50 p≤0.010 and day 60 p≤ 0.010).

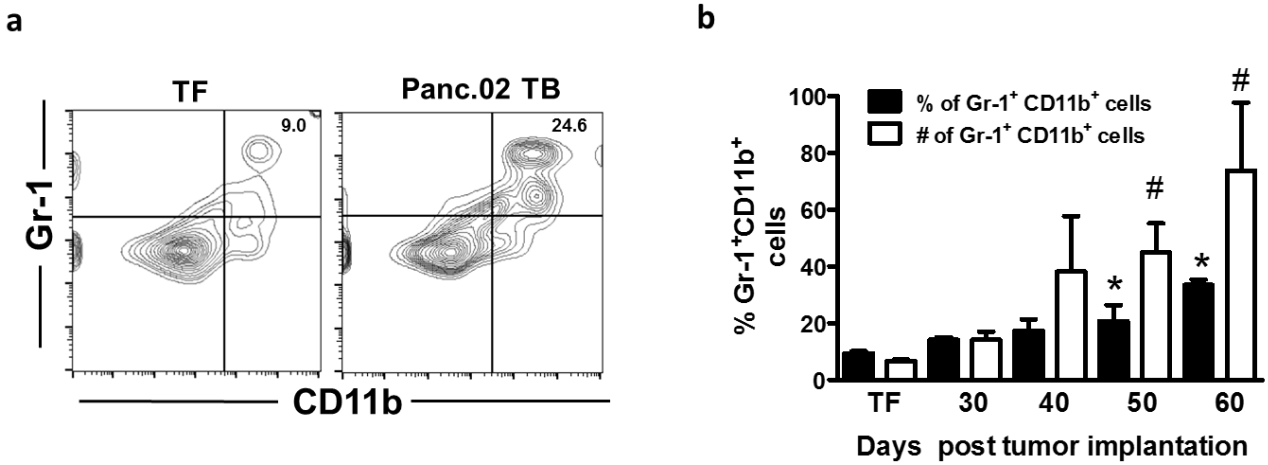

Fig. 3.3. Accumulation of Gr-1^{+}CD11b^{+} cells in Panc.02 tumor bearing mice. Flow cytometric analysis was performed on splenocytes at day 60-post tumor implantation. (a) Representative bivariate plot of the percentage of Gr-1^{+} vs. CD11b^{+} cells in TF (9.04%) and Panc.02 tumor bearing (24.6%) animals. (b) The number and percentage of MDSCs in the spleen of TF (n=8/group) and Panc.02 tumor-bearing animals at day 30 (n=2/group), 40 (n=7/group), 50 (n=9/group) and 60 (n=5/group) days post tumor implantation. The number of MDSCs was significantly higher than TF animals at day 50 and 60 post tumor implantation (b; Kruskal-Wallis test; κ=15.73, Dunn's Multiple Comparison Test: day 50 $p \leq 0.010$; day 60 $p \leq 0.010$). Number sign indicates a significant difference in number of MDSCs in Panc.02 animals in comparison to TF controls. The percentage of MDSCs was significantly higher than TF animals at days 50 and 60 post tumor implantation (b; Kruskal-Wallis test; κ=14.90, Dunn's Multiple Comparison Test: day 50 $p \leq 0.050$; day 60 $p \leq 0.001$). Asterisk indicates a significant difference in percentage of MDSCs in Panc.02 animals in comparison to TF controls.

3.3.4. Gr-1^{Hi}CD11b^{+} cells accumulate in Panc.02 tumor bearing animals.

A representative bivariate plot of Gr-1 vs. CD11b expression (Fig. 3.4a) and Ly6C vs. Ly6G expression (Fig. 3.4b) in the same TF or Panc.02 tumor-bearing animal is shown. Roman numerals indicate different subpopulations of MDSCs using both the Gr-1/CD11b and CD11b/Ly6G/Ly6C phenotypic marker designation. The MDSC subpopulation that is Gr-1^{Hi}CD11b^{+} (Fig. 3.4a) or CD11b^{+}Ly6G^{+}Ly6C^{Lo} (Fig. 3.4b) in TF and Panc.02 TB mice is denoted with (i). The MDSC subpopulation that is Gr-1^{Lo}CD11b^{+} (Fig. 3.4a) or CD11b^{+}Ly6G^{-} Ly6C^{Hi} (Fig. 3.4b) in TF and Panc.02 TB mice is denoted with (ii). There is a similar trend in the accumulation of both MDSC subsets in tumor bearing animals regardless of whether the Gr-1/CD11b or the CD11b/Ly6G/Ly6C markers are used (Fig. 3.4c and 3.4d, respectively). However, in this pancreatic tumor model, mice accumulated more Gr-1^{Hi}CD11b^{+} (Fig. 3.4c, $p<0.001$) or CD11b^{+}Ly6G^{+}Ly6C^{Lo} MDSCs (Fig. 3.4d, $p=0.001$).

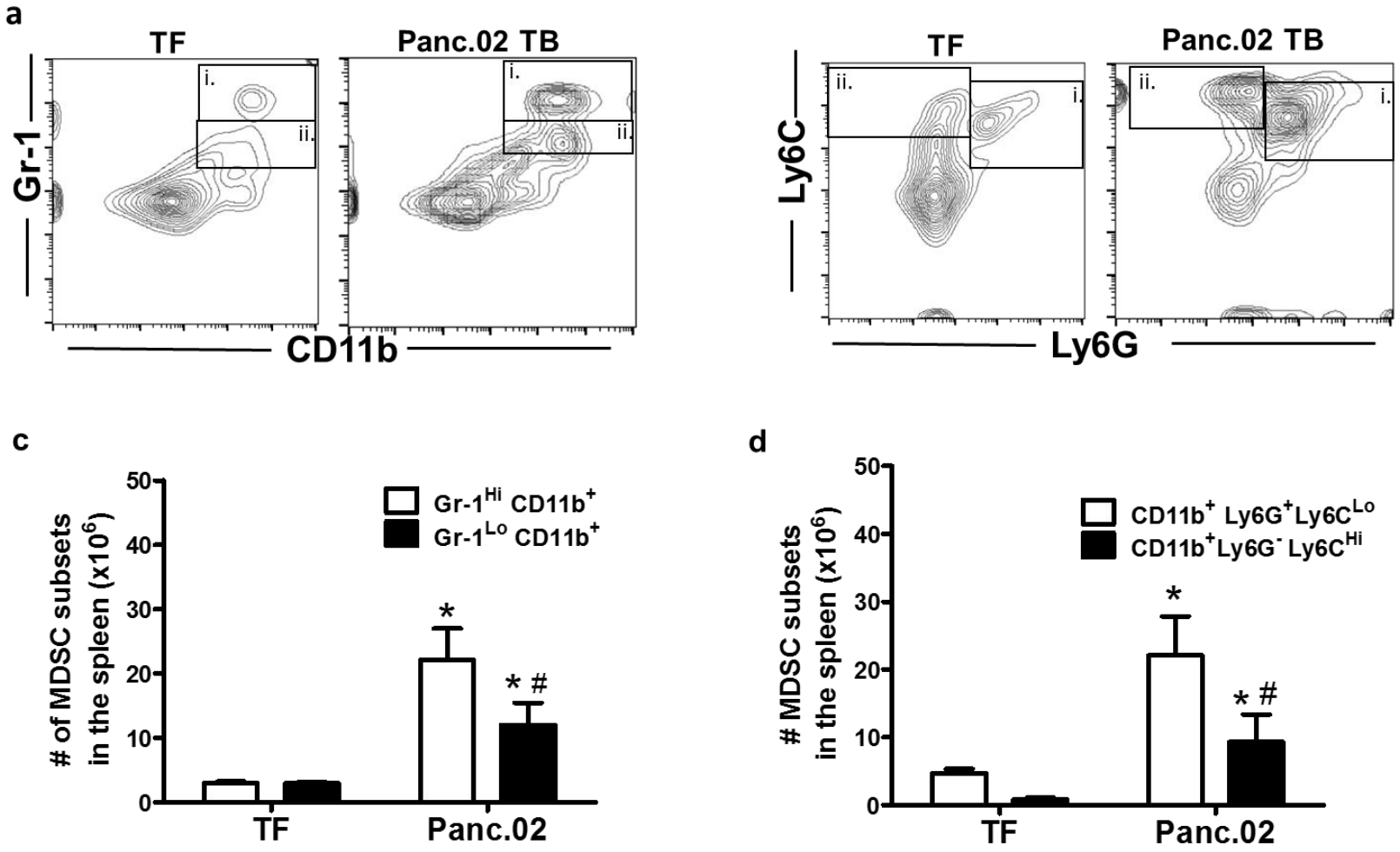

Fig. 3.4. Accumulation of MDSCs subsets in Panc.02 tumor bearing animals. Flow cytometric analysis was performed on splenocytes at day 60-post tumor implantation. Representative bivariate plots of Gr-1 vs. CD11b expression and Ly6C vs. Ly6G expression are shown in Figure 4a & 4b respectively. Roman numerals indicate

different subpopulations of MDSCs in TF and Panc.02 tumor-bearing animals. (i.) Indicates subpopulations that are Gr-1^{Hi}CD11b^{+} or CD11b^{+}Ly6G^{+}Ly6C^{Lo} in TF and Panc.02 TB mice (a & b). (ii.) Denotes subpopulations that are Gr-1^{Lo}CD11b^{+} or CD11b^{+}Ly6G^{-} Ly6C^{Hi} in TF and Panc.02 TB mice (a & b). (c) Number of Gr-1^{Hi}CD11b^{+} and Gr-1^{Lo}CD11b^{+} cells in TF (n=8/group) and Panc.02 (n=8/group) tumor bearing animals. A significant main effect of the tumor was observed (c; two-way ANOVA; $F_{(1, 28)}$=22.37, p<0.001). Bonferroni post hoc test showed a significant difference between Gr-1^{Hi}CD11b^{+} and Gr-1^{Lo}CD11b^{+} cells (c; two-way ANOVA; p<0.050) in Panc.02 animals. (d) Number of CD11b^{+}Ly6G^{+}Ly6C^{Lo} and CD11b^{+}Ly6G^{-} Ly6C^{Hi} cells in TF (n=8/group) and Panc.02 tumor bearing (n=8/group) animals. A significant main effect of tumor was observed (d; two-way ANOVA; $F_{(1, 28)}$= 13.10, p=0.001). Bonferroni post hoc test showed a significant difference between CD11b^{+}Ly6G^{+}Ly6C^{Lo} and CD11b^{+}Ly6G^{-}Ly6C^{Hi} MDSCs (d; two-way ANOVA; p<0.050) in Panc.02 animals. Asterisk indicates a significant difference in number of MDSCs in Panc.02 animals in comparison to TF controls. Number sign indicates a significant difference in number of MDSCs subsets in Panc.02 animals.

3.3.5. MDSCs from Panc.02 tumor bearing mice produce immunosuppressive factors TGF-β, IL-6 and IL-10.

TGF-β (Fig. 3.5a), IL-6 (Fig. 3.5b) and IL-10 (Fig. 3.5c) secretion from MDSC subsets alone, and in in the presence of T cells and APCs were quantified. The presence of either MDSC subset in the culture resulted in elevated TGF-β, IL-6, and IL-10; however, this was not significantly different than cultures containing T cells and APC alone.

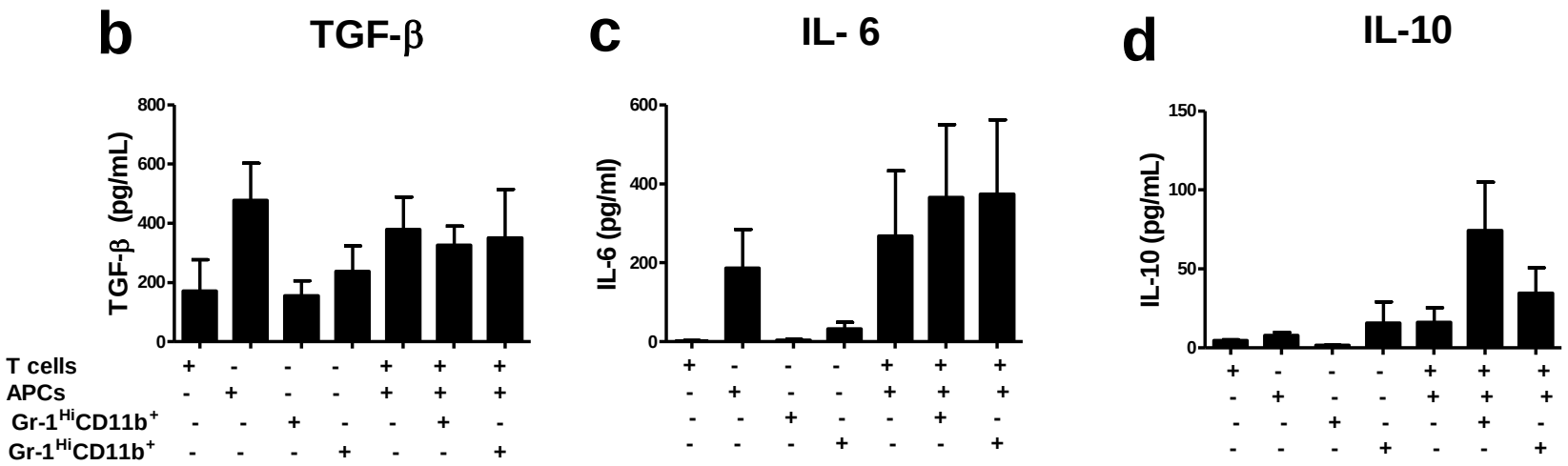

Fig. 3.5. Production of immunosuppressive mediators from Panc.02 MDSC subsets. MDSC subsets were isolated from Panc.02 tumor bearing animal's day 60-post tumor implantation and mixed with T cells isolated from non-tumor bearing BALB/c mice. Cells were incubated for 5 days and proliferation was measured via thymidine incorporation. (a) TGF-β, (b) IL-6, and (c) IL-10 secretion was measured in supernatants from MLR in Panc.02 tumor bearing animals (Fig. 5, n=5).

3.3.6. MDSCs from Panc.02 tumor bearing mice suppress T cell proliferation.

To test the ability of MDSC subsets to inhibit T cell proliferation, T cells from BALB/c mice were cultured with APCs from Panc.02 tumor bearing mice in a mixed lymphocyte reaction (MLR) with and without MDSC subsets from Panc.02 tumor bearing mice. Both MDSC subsets significantly suppressed the proliferative capacity of T cells (Fig. 3.6a). Gr-1^{Hi}CD$11b^{+}$ (CD$11b^{+}$Ly$6G^{+}$Ly$6C^{Lo}$) MDSCs inhibited T cell proliferation by 33.4± 10.2% (Fig. 3.6a, p≤0.050) and Gr-1^{Lo}CD$11b^{+}$ (CD$11b^{+}$Ly$6G^{-}$ Ly$6C^{Hi}$) MDSCs inhibit T cell proliferation by 29.1±11.0% (Fig. 3.6a, p≤0.050). To further characterize MDSC subsets from TF and Panc.02 tumor-bearing animals we measured gene expression of the immunosuppressive factors iNOS, ARG-1, TGF-β, IL-10, and IL-6 and the transcription factors STAT1 and STAT3 in MDSC subsets isolated from TF and Panc.02 tumor bearing animals by RT-PCR (Fig. 3.6a). Expression levels of NOS, STAT1, and STAT3 were significantly elevated in both MDSC subsets from Panc.02 tumor bearing animals in comparison to TF controls. Levels of TGF-β were significantly elevated in the granulocytic subset, and levels of ARG-1 and IL-10 were significantly elevated in monocytic subsets in comparison to TF animals. Gr-1^{Hi}CD$11b^{+}$ MDSCs expressed higher levels of iNOS and TGF-β in comparison to Gr-1^{Lo}CD$11b^{+}$ MDSCs. In contrast Gr-1^{Lo}CD$11b^{+}$ MDSCs had elevated expression of ARG-1, STAT1, STAT3, and IL-10 in comparison to Gr-1^{Hi}CD$11b^{+}$ MDSCs.

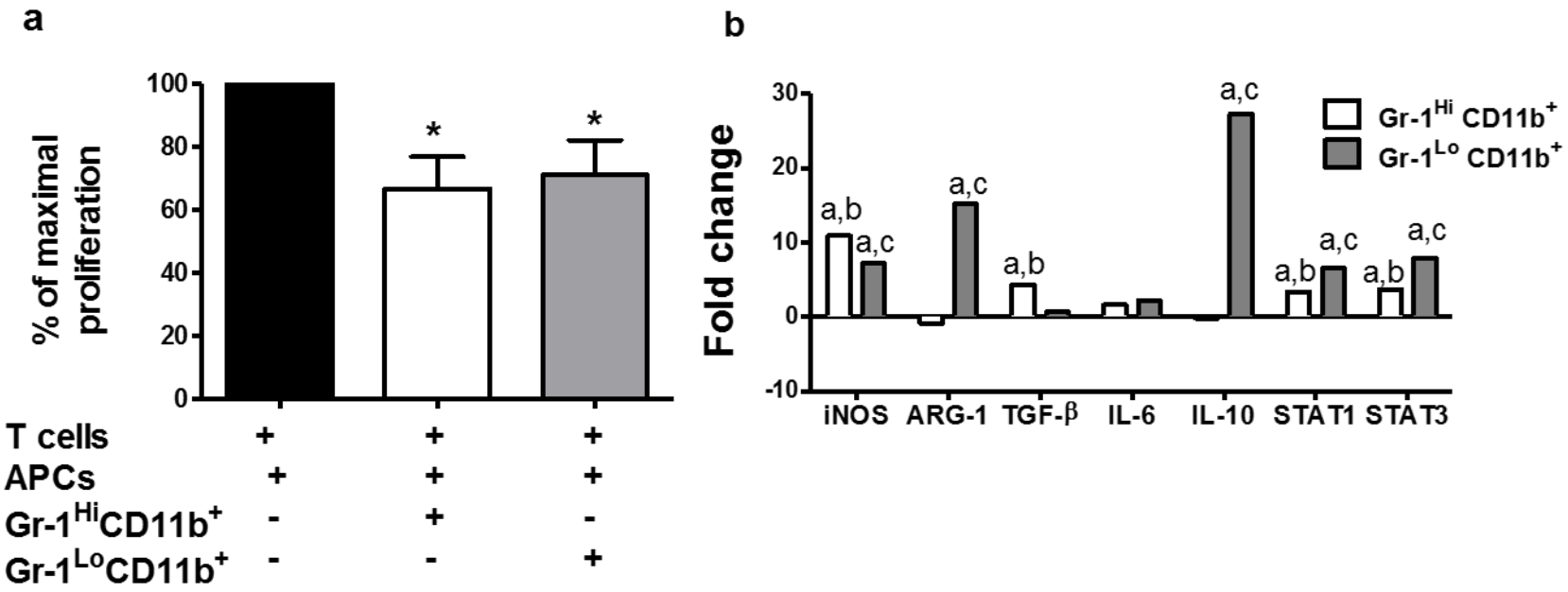

Fig. 3.6. Suppression of T cell proliferation and gene expression of immunosuppressive mediators and transcription factors in MDSCs from Panc.02 tumor bearing animals. (a) Irradiated APCs (2000 Rads) and MDSC subsets were isolated from Panc.02 tumor bearing animal's day 60-post tumor implantation and mixed with T cells isolated from non-tumor bearing BALB/c mice. Cells were incubated for 5 days and proliferation was measured via thymidine incorporation. Percent of maximal proliferation (i.e. proliferation of T cell plus APCs alone) is reported for animals with tumor volumes larger than $1cm^3$. Asterisk indicates a significant difference from T cells plus APCs (a; one-way ANOVA, $F_{(2, 18)}=4.40$). A significant difference was observed between T Cells + APCs alone and Gr-1^{Hi}CD11b^+ (CD11b^+Ly6G^+Ly6C^{Lo}) and Gr-1^{Lo}CD11b^+ (CD11b^+Ly6G^-Ly6C^{Hi}) MDSC subsets Bonferroni multiple comparison $p \leq 0.050$. MDSCs subsets alone did not proliferate (data not shown). (b) Gene expression of the immunosuppressive factors iNOS, ARG-1, TGF-β, IL-10, IL-6 and the transcription factors,

STAT1 and STAT3 were measured in MDSC subsets isolated from TF and Panc.02 tumor bearing animals by RT-PCR. Expression levels were normalized to 18s RNA and Fold change was determined using the $2^{\Delta\Delta CT}$ method. Fold change greater than 3 was considered significant. Expression levels of iNOS, STAT1, and STAT3 were significantly elevated in both MDSC subsets from Panc.02 tumor bearing animals in comparison to TF controls. Levels of TGF-β were significantly elevated in the Gr-1^{Hi}CD11b^{+} (granulocytic) subset, and levels of ARG-1 and IL-10 were significantly elevated in the Gr-1^{Lo}CD11b^{+} (monocytic) subset in comparison to TF animals. Gr-1^{Hi}CD11b^{+} MDSCs expressed higher levels of iNOS and TGF-β in comparison to Gr-1^{Lo}CD11b^{+} MDSCs. In contrast Gr-1^{Lo}CD11b^{+} MDSCs had elevated expression of ARG-1, STAT1, STAT3, and IL-10 in comparison to Gr-1^{Hi}CD11b^{+} MDSCs. MDSCs were isolated with greater than 90% purity (data not shown). a indicates a difference from TF animals, b indicates a difference from Gr-1^{Lo}CD11b^{+} MDSCs, and c indicates a difference from Gr-1^{Hi}CD11b^{+} MDSCs.

3.3.7. Contribution of MDSCs to Panc.02 tumor growth.

The number of splenic Gr-1$^+$CD11b$^+$ cells in Panc.02 tumor-bearing animals was significantly correlated with tumor volume at sacrifice (Fig. 3.7a; Spearman correlation ρ=0.409, p=0.014). In addition, total splenocyte number was significantly correlated with tumor volume at sacrifice (data not shown; ρ=0.5440, p<0.001). To determine the contribution of MDSC accumulation to Panc.02 tumor progression, we examined whether depletion of this myeloid population would reduce tumor growth. Depletion of Gr-1$^+$ cells significantly retarded Panc.02 tumor progression compared to PBS and isotype mAb treated mice (Fig. 3.7b, p=0.002).

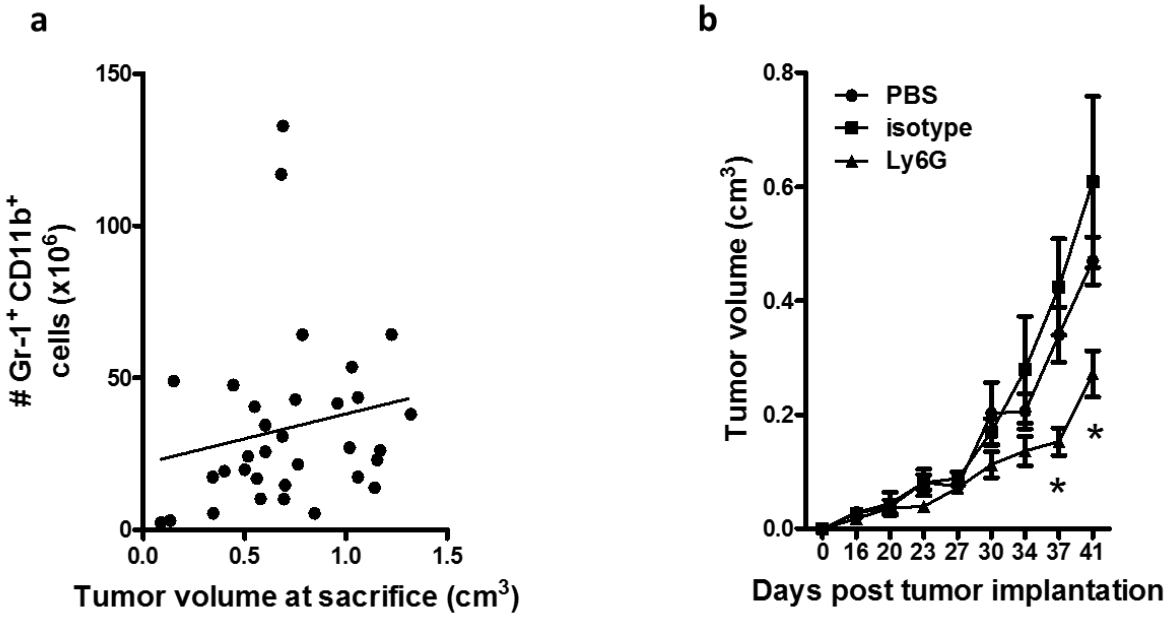

Fig. 3.7. Contribution of MDSCs to Panc.02 tumor growth. (a) Spearman correlation was used to determine if there was a positive relationship between MDSC accumulation and Panc.02 tumor growth. The correlation between the number of Gr-1$^+$CD11b$^+$ cells in spleen and tumor volume at sacrifice (a; ρ=0.409, p≤0.010, n=33). (b) Antibody depletion of Gr-1$^+$ cells was used to determine the impact of MDSCs to Panc.02 tumor burden. A significant treatment X time interaction (b; two-way ANOVA, $F_{(16,264)}$=2.47, p=0.002) was observed in Panc.02 mice receiving PBS (n=10/group), isotype (n=10/group) and Ly6G (n=13/group) treatments. Bonferroni multiple comparison test showed a significant reduction in tumor volume in Ly6G treated mice in comparison to PBS and isotype control animals at day 37 and 41 post tumor implantation (b; Bonferroni multiple comparison test: Ly6G vs. PBS at day 37 and 41, p<0.050: Ly6G vs. isotype day 37, p<0.001 and day 41, p≤0.001).

3.3.8. Tumors from Panc.02 tumor bearing mice express immunosuppressive mediators and transcription factors that drive MDSC accumulation and suppressive function.

Gene expression of immunosuppressive factors (ARG-1, iNOS, Cox-2, TGF-β, IL-6, and IL-10), CD3 and the transcription factors, STAT1 and STAT3, were quantified in cultured Panc.02 cells, and in tumors from Panc.02 mice (n=13). The immunosuppressive factors ARG-1, iNOS, Cox-2, IL-6, and IL-10 and were significantly elevated in the explanted tumor in comparison to cultured

pancreatic tumor cells. The transcription factors, STAT1 and STAT3, and T cells were also significantly elevated in the tumors of mice in comparison to cells alone.

Table 3.1. Characterization of immunosuppressive factors, immune cells, and transcription factors in tumors from Panc.02 mice.

Table 3.1. Tumor fold change in comparison to Panc.02 cells		
	Experiment 1	**Experiment 2**
ARG-1	21.41	41.52
iNOS	9.73	8.45
Cox-2	-8.09	-20.20
TGF-β	-1.33	-2.01
IL-6	6.69	8.13
IL-10	6.40	24.17
STAT1	47373.26	59599.42
STAT3	1.32	1.36
CD3	1389.85	2335.27

3.5. Discussion

We demonstrated that a reduction in tumor-induced MDSC accumulation via antibody depletion of Gr-1^{+} MDSCs significantly retarded Panc.02 tumor growth. These data suggest an important role for MDSC-induced immune suppression in pancreatic tumor progression. Furthermore, we have shown that MDSCs accumulate over time, with a preferential accumulation of the Gr-1^{Hi}CD11b^{+} (CD11b^{+}Ly6G^{+}Ly6C^{Lo}) MDSC subset in tumor bearing mice. This accumulation of MDSCs was correlated with tumor volume, and occurred concomitantly with increased plasma levels of GM-CSF, IL-6, TNF-α, MCP-1, MIP-1α, VEGF, and TGF-β in Panc.02 tumor bearing mice. Both MDSCs subsets from tumor bearing mice inhibited T cell proliferation and produced immunosuppressive factors TGF-β, IL-6, and IL-10 in culture. Thus, the Panc.02 tumor model is an appropriate system to evaluate the role of MDSC-induced immune suppression in pancreatic tumor progression.

The role of the immune system in pancreatic cancer progression is well documented (2, 135, 136). Tumors exploit a variety of mechanisms to suppress host immune responses. Antigen-specific T cell tolerance is one of the major mechanisms by which tumors escape immune cell destruction (139-141). Several populations of cells have been identified with immune-suppressive function, including MDSCs (14). MDSCs are a potent immunosuppressive cell population with the ability to suppress both innate and adaptive immune responses (180). Thus they contribute to disease progression by impairing host antitumor immunity. Suppressive mechanisms utilized by MDSCs include L-arginine

depletion in the tumor microenvironment, depletion/deprivation of cysteine, and induction of Tregs (15). MDSCs can also disrupt/down regulate CD3ζ signaling and produce peroxynitrite leading to nitration of the T cell receptor which results in reduced signaling from T cells (17). MDSCs produce TGF-β (181) and IL-10 (21) which both have the ability to suppress anti-tumor T cell responses. The suppressive activity of MDSCs on T cells requires contact and can be antigen specific or nonspecific (12). Thus, multiple lines of evidence indicate that MDSC are robust inhibitors of anti-tumor immune mechanisms (15).

MDSCs are elevated, and correlated with cancer stage in breast, colon, lung, renal, esophageal, gastric, melanoma, prostate colon and pancreatic cancer patients in comparison to normal volunteers (24). Gabitass and colleagues show elevated circulating MDSCs in pancreatic, esophageal, gastric and upper GI cancer patients in comparison to controls. Increases in MDSCs are associated with 22% increased risk of death (hazard ratio= 1.22: 95% CI, 1.06-1.41) in cancer patients in comparison to healthy controls (25). Similarly, MDSCs accumulate in pancreatic cancer patients, and their accumulation correlates with disease progression (27, 28) and a concurrent reduction in $CD8^{+}$T cells (28). Granulocytic MDSCs ($Lin^{-}HLA^{-}DR^{-}CD33^{+}CD11b^{+}CD15^{+}$) are significantly elevated in the blood of pancreatic cancer patients in comparison to healthy donors (169). These results demonstrate that MDSCs are important mediations of immune suppression in pancreatic cancer patients.

MDSCs have also been documented in murine models of pancreatic cancer including transgenic (30, 31), orthotopic (32) and subcutaneous models

(38) of pancreatic cancer. We observed that MDSC accumulation in Panc.02 tumor bearing mice is time dependent; and correlates with tumor volume. These data suggests that factors coming from the tumor are supporting the growth/expansion of MDSCs in this tumor model. This is relevant because the Panc.02 tumor model is characterized by elevated inflammatory factors thus, MDSCs may not accumulate to the same extent in tumor models with less of an inflammatory profile. Murine MDSCs are comprised of two subsets, Gr-$1^{Hi}CD11b^{+}$ ($CD11b^{+}Ly6G^{+}Ly6C^{Lo}$) cells or granulocytic MDSCs and Gr-$1^{Lo}CD11b^{+}$ ($CD11b^{+}Ly6C^{Hi}Ly6G^{-}$) or monocytic MDSCs. A tumor-induced accumulation of MDSCs was evident and comparable using either the Gr-1/CD11b or the CD11b/ Ly6C/Ly6G nomenclature. Specifically, we demonstrated that MDSCs comprise 30% of the spleen by day 60 post tumor implantation, and that this is due to a preferential accumulation of the granulocytic subset of MDSCs. Previous studies have examined MDSC subsets expansion in lymphoma, lung, melanoma, colon, and breast tumor models where 70-80% of the MDSC expansion was attributed to accumulation of the granulocytic subset and 20-30% of the expansion was due to the monocytic subset (12). Khaled *et al.* show a greater expansion of granulocytic MDSCs in comparison to monocytic MDSCs in pancreatic cancer patients. Thus our results with respect to MDSC accumulation in the Panc.02 tumor model are similar to previous reported studies (12, 182, 183).

MDSCs expand in response to various growth factors, and inflammatory mediators (12). Levels of VEGF, GM-CSF, IL-1β, IL-6, and TGF- β are implicated

in the expansion and activation of MDSCs in animal models in and cancer patients. Tumor derived VEGF and GM-CSF cause the expansion of MDSCs in melanoma, and lymphoma murine cancer models (184). IL-1β and IL-6 are also implicated in the accumulation of MDSCs in a murine mammary carcinoma model (16, 19). IL-6 causes recruitment of MDSCs with metastatic capacity in a murine breast cancer model (168). TGF- β is similarly implicated in the expansion of MDSCs in murine mammary carcinoma models (185). Treatment of PBMCs with various cytokine (e.g. TNF-α, VEGF, IL-6 and GM-CSF) generated MDSCs with the capacity to inhibit T-cell proliferation and IFN-γ production *in vitro* from the PBMCs of healthy donors (186). However, the combination of IL-6 and GM-CSF generated MDSCs with the greatest suppressive capacity (186). We observed a significant increase in plasma levels of inflammatory mediators and growth factors GM-CSF, IL-6, MCP-1, MIP-1α, TNF-α, VEGF and TGF-β, in our Panc.02 tumor bearing animals in comparison to TF controls. It is plausible that the MDSC accumulation we observed in the current study is a direct result of increased plasma levels of these inflammatory mediators and growth factors.

The contribution of MDSCs on tumor growth has been studied in numerous tumor models. Suzuki *et al.* reports that gemcitabine reduces the number of MDSCs in the spleen without altering the number of effector cells ($CD4^+$ and $CD8^+$ T cells) in lung and mesothelioma tumor models (153). 5FU reduces splenic and tumor MDSCs without altering the number of B cells, T cells and DCs. In addition, tumor-bearing mice (lymphoma) treated with 5FU have restored $CD8^+$ T cell function (172). A previous study also demonstrated that

MDSC reduction via gemcitabine in combination with a dendritic cell vaccination induced immunity in the Panc.02 tumor model (145). Another study utilizing a genetically engineered mouse model of PDAC demonstrated that targeted depletion of the granulocytic MDSC subset increases the accumulation of $CD8^+$ T cells at the tumor site and induces of apoptosis of tumor cells (171). Sildenafil treatment reduces MDSC accumulation at the tumor site, and deceases serum levels of IL-6 and VEGF (41). IL-6 and VEGF are known factors that drive the expansion of MDSCs (12, 19). This consequent reduction of MDSCs in the tumor also restored activation of T cells in the tumor. We have shown antibody depletion of MDSCs reduced tumor burden in Pan.02 mice (Figure 3.7b). These data provide a direct link for MDSCs in enhancing tumor burden, and contributing to the suppression of anti-tumor immune mechanisms in a murine pancreatic tumor model.

The inhibition of T cell function is one mechanism by which MDSCs can inhibit anti-tumor immunity (15). In our study, both MDSC subsets inhibited T cell proliferation by nearly 30%. The activation of these immature myeloid cells in disease states such as infection and cancer results in the up regulation of numerous immunosuppressive factors, e.g. ARG-1, iNOS, and ROS (12). To further characterize MDSC subsets from TF and Panc.02 tumor-bearing animals we measured gene expression of the immunosuppressive factors iNOS, ARG-1, TGF-β, IL-10, and IL-6. MDSC functional capacity is hypothesized to be subset specific and mediated through STAT1 and STAT3 differentially. Thus we measured gene expression of transcription factors, STAT1 and STAT3 in MDSC

subsets isolated from TF and Panc.02 tumor bearing animals. We demonstrated that gene expression of iNOS, STAT1, and STAT3 were significantly elevated in both MDSC subsets from Panc.02 tumor bearing animals in comparison to TF controls. Levels of TGF-β were significantly elevated in the granulocytic subset, and levels of ARG-1 and IL-10 were significantly elevated in monocytic subsets in comparison to TF animals. Gr-$1^{Hi}CD11b^{+}$ MDSCs expressed higher levels of iNOS and TGF-β in comparison to Gr-$1^{Lo}CD11b^{+}$ MDSCs. In contrast Gr-$1^{Lo}CD11b^{+}$ MDSCs had elevated expression of ARG-1, STAT1, STAT3, and IL-10 in comparison to Gr-$1^{Hi}CD11b^{+}$ MDSCs. In a murine lymphoma tumor model, granulocytic MDSCs have higher levels of ROS (162) and lower levels of NO mediated through STAT3-dependent mechanism (12). In contrast, the monocytic MDSCs have higher levels of iNOS (12) and lower levels of ROS mediated through STAT1 in a murine lymohoma tumor model (163). Gabrilovich *et al.* reports the monocytic MDSCs express ARG-1, and iNOS, via a STAT1 dependent mechanism; whereas granulocytic MDSCs express ARG-1 and ROS via STAT3 mechanisms. We observed similar expression of iNOS between both MDSC subsets. We also showed that ARG-1 was down regulated in granulocytic MDSCs and up regulated in monocytic MDSCs. Additionally, both MDSC subsets express STAT1 and STAT3. Overall these data demonstrate that both MDSCs subsets express immunosuppressive factors, which may aid the immune suppression observed in pancreatic cancer patients and potentiate pancreatic cancer growth.

In conclusion, we characterized the dynamics of MDSC subset accumulation during tumor development, and the functional properties of MDSC subsets in the Panc.02 model. We also demonstrated that MDSCs directly contribute to pancreatic tumor growth. Results from the current study provide insight into potential mechanism by which MDSCs are enhancing tumor growth in this murine tumor model.

Supplementary Table 1. Taqman gene expression Assay ID for primer/probe sets

Supplementary Table 1. Taqman gene expression Assay ID for primer/probe sets	
Primer/probe set	**Assay ID**
IL-6	Mm00446190_m1
IL-10	Mm00439614_m1
STAT 1	Mm00439531_m1
STAT 3	Mm01219775_m1
ARG-1	Mm00475988_m1
iNOS	Mm00440502_m1
18S RNA	Mm03928990_g1
TGF-β	Mm01178820_m1
CD3	Mm00599684_g1
Cox-2	Mm00478374_m1

CHAPTER 4

Obesity accelerates pancreatic tumor growth and increases the accumulation of Gr-1^{+}CD11b^{+} myeloid derived suppressor cells

4.1. Abstract

Obesity is associated with an increased risk and reduced survival from many cancer types, including pancreatic cancer. Obesity-induced changes in metabolic and inflammatory mediators are proposed as biological mediators underlying this relationship. However, an obesity-induced change in immunity and the emergence of immunosuppressive cells has not been examined. The goal of the current study was to determine if obesity altered the accumulation and function of MDSCs in a murine model of pancreatic cancer. C57BL/6 mice were placed on a 30% kcal carbohydrate calorie restricted diet, a 10% kcal from fat diet, and a 60 kcal% from fat diet fed *ad libitum* for 16 weeks prior to generate lean, control and obese mice, respectively. After 16 weeks on the respective diets, mice were injected with $1x10^6$ Panc.02 cells s.c. and monitored starting at day 13 post-tumor implantation until 60 days post tumor implantation, or until tumor reached the ethical limit. Obesity significantly enhanced tumor growth and reduced survival in comparison to lean and control mice. Obesity increased accumulation of total ($Gr\text{-}1^{+}CD11b^{+}$), granulocytic ($Gr\text{-}1^{Hi}CD11b^{+}$) and monocytic ($Gr\text{-}1^{Lo}CD11b^{+}$) MDSCs in lymphoid organs of Panc.02 tumor bearing animals. MDSC subsets from all dietary groups suppressed T cell proliferation. Obesity enhanced gene expression of Gr-1 and immunosuppressive factors IL-10 and IL-6 in the adipose tissue in comparison to lean mice. These data suggest that obesity-induced MDSC accumulation enhances tumor growth and reduces survival in Panc.02 tumor-bearing mice.

4.2. Introduction

Pancreatic cancer is the fourth leading cause of cancer related deaths in the U.S. (1, 29). The current 5-year survival rate for pancreatic cancer patients is ~5% (1). Current risk factors for pancreatic cancer include smoking, diabetes, and obesity. Modifiable risk factors, such as obesity, are potential targets for a reduction in the incidence of pancreatic cancer. Obesity can increase risk and (43, 44) reduce survival (187) from pancreatic cancer. The mechanisms underlying the link between obesity and pancreatic cancer are not fully understood.

Obesity is characterized as a state of low-grade chronic inflammation. Obesity-associated inflammation is the result of macrophage infiltration in the adipose tissue. Expansion of the adipose tissue upon weight gain leads to the infiltration of macrophages through chemokines such as CCL2 (57). Various inflammatory mediators (e.g. IL-6, TNF-α, IL-1β), and adipokines (e.g. resistin, leptin and adiponectin) are secreted by adipocytes and macrophages and contribute to inflammation (57). This prolonged immune activation (chronic inflammation) contributes to the pathology of obesity associated diseases, including chronic pancreatitis and pancreatic carcinoma (188).

Numerous studies demonstrate that obesity can alter the innate and adaptive immune responses (46). Serrano *et al*. found that obesity increases risk of infection (e.g. pneumonia and wound infections) after trauma in comparison to normal weight individuals. Similarly, in clinical studies, obesity reduces the number of $CD4^+$ and $CD8^+$ T cells and impairs T cell proliferation in comparison

to normal weight patients (189). Obesity also decreases NK cell number, increases activating receptor NKG2D (190) and impairs function (e.g. cytotoxicity) of NK cells in comparison to normal weight individuals (191). These data demonstrate that obese individuals have increased risk of infection, slower wound healing, and decreased T cell and natural killer (NK) cell function (49, 75). Since the most potent anti-tumor responses originate from effector T cells ($CD8^+$ T cells) and NK cells (192, 193), these data suggest that obesity leads to impairment in anti-tumor immunity.

In addition to obesity-induced impairments in anti-tumor immunity, obesity may also increase the immunosuppressive environment associated with pancreatic cancer. In particular, the obesity-induced changes in inflammatory mediator may contribute to the emergence of MDSCs. MDSCs are a diverse population of immature macrophages, granulocytes, and dendritic cells (15). In pathological conditions, MDSCs expand in response to inflammatory mediators and tumor derived factors including PGE_2, IL-6, VEGF and GM-CSF (16, 23). MDSCs can suppress cells of both the innate and adaptive immune system including NK cell, macrophages, DCs, and T cells (12). In addition to altering innate and adaptive immunity, MDSCs alter anti-tumor immunity (12). However, it is not known if obesity alters the accumulation and function of MDSCs.

The goal of the current study was to determine if obesity altered tumor growth, survival, and MDCS accumulation and function in a murine model of pancreatic cancer. A better understanding of the effects of obesity on tumor-

derived immune suppressive mechanisms is critical to elucidate the mechanism underlying mechanisms linking obesity and pancreatic cancer.

4.3. Materials and Methods

4.3.1. Animals

Female 6-week-old C57BL/6 and BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, MA). All mice were housed at the Pennsylvania State University and maintained on a 12-hour light/dark cycle with free access to food and water. C57BL/6 mice were fed a 30% carbohydrate calorie restricted diet, a 10% kcal from fat diet, and 60% kcal from fat diet for 16 weeks to induce a lean, control, and obese phenotype, respectively. Mice continued on their respective diets following tumor implantation. All diets were purchased from Research Diets (New Brunswick, New Jersey). Food intake and body weight, were monitored as previously reported (173), and mice were observed daily for signs of ill health. Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals." The Institutional Animal Care and Use Committee of the Pennsylvania State University approved all animal experiments.

4.3.2. Tumor Cell Line and Cell Culture

The murine ductal adenocarcinoma cell line Panc.02 were originally established through the induction of pancreatic tumors with 3-methylcholanthrene and serial s.c. transplantation in C57BL/6 mice (176). Panc.02 cells were obtained from Dr. Jeffrey Schlom (National Cancer Institute, National Institute of Health) and cultured in McCoy’s 5A without G418 (Thermo Scientific; Logan, UT), and

supplemented with 10% FBS (Gemini Bio Products; Sacramento, CA), 0.1mM non-essential amino acids (Mediatech; Manassas, VA), 1mM sodium pyruvate (Mediatech), 2mM glutamine (Mediatech), 10mM HEPES (Mediatech), 100U/mL penicillin streptomycin (Mediatech). Panc.02 Cells were maintained at 37°C and 5% CO_2 and passaged every 3-4 days using trypsin/EDTA (Mediatech).

4.3.3. Tumor Protocol

Panc.02 cells ($1x10^6$) were suspended in PBS and injected subcutaneously (s.c.) into the lumbar region of lean, control and obese C57BL/6 mice. Tumor growth was monitored three times per week with a digital caliper from day 13 post tumor implantation until all mice reached the ethical limit for tumor size (1.5 cm^3) or day 60 post tumor implantation. Tumor volume was calculated by multiplying the short side ^{x} short side ^{x} long side /2 ^{x}0.001 to get tumor volume in cm^3. For survival studies, mice were sacrificed when tumor size reached the ethical limit for tumor size of 1.5cm^3, and were counted as death for time to event analysis (survival analysis).

4.3.4. Immunological assays

Isolation of immune cells

Spleens were harvested via gross dissection and splenocytes were prepared from individual mice by mechanical dispersion, as previously described (177). Briefly, harvested spleens were mechanically disrupted with a syringe plunger and passed through a 70 µm nylon mesh strainer (BD Biosciences; Bedford, MA), erythrocytes were lysed with ACK lysing buffer (Lonza), washed twice in complete medium (RPMI 1640 Mediatech), and supplemented with 10% FBS

(Gemini Bio Products), 0.1mM non-essential amino acids (Mediatech), 1mM sodium pyruvate (Mediatech), 2mM glutamine (Mediatech), 10mM HEPES (Mediatech), 100U/mL penicillin streptomycin (Mediatech). Cell counts and viability were determined via trypan blue exclusion.

MDSC Cell Isolation

MDSCs were isolated from a single cell suspension of splenocytes as per manufacturer's instructions (MSDC Isolation kit; Miltenyi Biotec; Gladbach, Germany). Cell count was determined and up to $1x10^8$ splenocytes were centrifuged for 10 minutes at 300g at 4°C. Supernatants were aspirated and cells were re-suspended in 350µL of isolation buffer (PBS, 0.5% BSA, and 2mM EDTA). FcR blocking reagent (50µL) was added, vortexed, and incubated for 10 minutes in the refrigerator 4°C. After incubation 100µL of anti-Ly-6G-antibody was added to the cells, mixed well, and incubated for 10 minutes at 4°C. Cells were washed by adding 10mL of buffer and centrifuging at 300g for 10 minutes at 4°C. The cells were re-suspended in 800µL of buffer, then 200µL of anti-biotin microbeads were added, mixed well, and incubated for 15 minutes at 4°C. Cells were washed by adding 10-20mL of buffer and centrifuging at 300xg for 10 minutes at 4°C. Aspirated supernatant off completely and the cell pellets were then resuspended in 500µL of buffer.

For magnetic separation with columns, LS columns were rinsed with 3mL of buffer and cell suspensions were added onto the column. Columns were washed three times with 3mL of buffer and the unlabeled cells that pass through were collected. The flow-through containing unlabeled cells was collected and placed

on ice and labeled as theGr-1 ^{dim} Ly-6G^{-} cell fraction. The cells remaining on the column were collected by removing the columns from the magnet and flushing the columns with buffer. The collected cells were labeled as the Gr-1^{high} Ly-6G^{+} Granulocytic MDSC cells.

For further Isolation of the Gr-1^{dim} Ly-6G^{-}cells, the remaining cell suspensions were centrifuged at 300xg for 10 minutes at 4°C. The pellet was re-suspended in 400µL of buffer and 100µL of anti-Gr-1-antibody was added, mixed well, and incubated for 10 minutes at 4°C. Cells were washed by adding 5-10mL of buffer centrifuging at 300xg for 10 minutes at 4°C and the supernatant were aspirated. The cells were re-suspended in 900µL of buffer and 100µL of streptavidin microbeads were added, mixed well, and incubated for 15 minutes at 4°C. Cells were washed by adding 10-20mL of buffer and centrifuging at 300xg for 10 minutes at 4°C and the supernatants were aspirated completely. The cells were re-suspended in 500µL of buffer and magnetic separation was performed.

The MS column was placed in the magnetic field of a suitable MACS Separator and the column was prepared by rinsing with 500µL of buffer. The cell suspension was applied onto the column. The unlabeled cells passed through the column. The column was rinsed 3 times with 500µL of buffer. The columns were removed from the magnet and flushed with buffer to collect the Gr-1 ^{dim}Ly-6G^{-} Monocytic MDSC fraction. After isolation MDSC subsets were counted and viability determined via trypan blue exclusion.

T cell and APC Isolation

T cells were isolated via negative selection using the Dynabeads® Untouched^{TM}Mouse T cells Kit (Life Technologies). APCs were isolated using the Dynabeads® Mouse Pan T (Thy1.2) Kit (Life Technologies). After isolation, T cells and APCs were counted and viability determined via trypan blue exclusion.

Flow cytometric analyses

Single cell suspensions of splenocytes were washed twice in PBS at 4°C. Cells were incubated with Fc block (Biolegend) and $1x10^6$ cells were stained with saturating concentrations of conjugated antibodies including CD19 FITC (BD Biosciences), CD4 PE (BD Biosciences), CD8 APC (BD Biosciences), CD3 APC-Cy7 (BD Biosciences), F4/80 APC (BD Biosciences), Gr-1 FITC (BD Biosciences), and CD11b PE (BD Biosciences) for 30 min at 4°C, as previously described (177). Following incubation with the conjugated antibodies, cells were washed twice in PBS and fixed in 1% paraformaldehyde. Lymphoid and myeloid cells were gated on forward vs. side scatter and a total of 30,000 events. All flow cytometric analyses were performed on a Beckman Coulter FC500 flow cytometer (Beckman Coulter; Indianapolis, IN). Flow cytometric analyses were plotted and analyzed using Flow Jo software (Tree Star; Ashland, OR).

Mixed Lymphocyte Reaction (MLR)

Isolated splenic T cells ($1x10^5$) from BALB/c animals were co-cultured with irradiated (2000 rads) APCs ($5x10^5$) isolated from lean, control, and obese Panc.02 tumor-bearing C57BL/6 mice and either granulocytic (Gr-1^{high}Ly6G$^+$) or monocytic (Gr-1^{dim}Ly6G$^-$) MDSCs ($5x10^4$). Cells were incubated in flat-bottomed,

96-well plates and were pulsed with 1μCi per well of tritiated thymidine at 72 hours. Proliferation was assessed by tritiated thymidine (H^3) incorporation at 96 hours (Perkin Elmer; Waltham, MA) on a microbeta plate reader (Perkin Elmer; Waltham, MA). Each assay was performed in triplicate.

Cytokine Measurements

Cytokines, including G-CSF, GM-CSF, IL-1β, IL-6, KC, MCP-1, MIP-1α, and TNF-α, were measured in mouse plasma of tumor free (TF) and Panc.02 tumor bearing lean, control, and obese mice using the MILLIPLEX MAP Cytokine kit (Millipore; Billerica, MA). Mean fluorescence intensity was calculated from duplicate samples using the luminex-200 software and quantified on the Bio-Plex™ 200 System (Bio-Rad; Hercules, CA). TGF-β was assessed in plasma of TF and Panc.02 tumor-bearing mice among all dietary groups via ELISA (Biolegend) per manufacturer's instructions. Cytokines were quantified on an Epoch Micro-plate Spectrophotometer (Biotek; Winooski, VT). Each assay was performed in duplicate.

Gene Expression

Gene expression of ARG-1, iNOS, Cox-2, TGF-β, IL-6, IL-10, CD3, STAT1, STAT3 and Gr-1 was assessed in the adipose tissue and tumor tissue of lean, control, and obese Panc.02-tumor bearing mice. Total RNA was extracted and genomic DNA contamination was removed by using RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was quantified by using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), and reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Life

Technologies). Real-time qPCR was performed by using TaqMan real time PCR reagents (Life Technologies and an Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies). Primer sequences were based on published studies and actual primer sequences were listed in Supporting Information Table 1. Real-time qPCR data were calculated using the standard curve method and normalized to 18s RNA. Relative quantification or fold change in gene expression was determined using the $2^{\Delta\Delta Ct}$ method using data from lean mice as reference control (178).

Immunofluorescence

Adipose tissue was harvested from lean, control, and obese Panc.02 tumor-bearing mice, placed on O.C.T. compound (Sakura; Torrance, CA), and flash frozen in liquid nitrogen. O.C.T. (Sakura; Torrance, CA) sections (10μm) were mounted on super frost plus glass slides (Thermofisher Sci, Grand Island, NY) and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Sections were blocked with antibody diluent (BD Biosciences) for one hour at room temperature and washed 3X with 0.1% Tween-PBS wash buffer for 5 minutes. Primary antibodies Gr-1 (Alexa-647), and CD11b (FITC) from (BD) were diluted in antibody diluent and incubated in a humidified container overnight at 4°C. Sections were washed 3X with Tween-PBS wash buffer. DAPI (Sigma-Aldrich) in PBS was added to slides (1gμ/mL) for 5 minutes at room temperature. Sections were washed 3X in wash buffer and mounted with Fluormount-G (eBioscience) and stored at 4°C. Hematoxylin and eosin (H&E) staining of adipose tissue sections from lean, control, and obese mice were prepared with

the Gemini automated staining system. All slides were imaged on the Keyence BZ-9000E fluorescence microscope (Itasca, IL).

4.3.5. Statistics

All data are presented as the mean plus or minus the standard error mean. Differences in food intake, body weight, and tumor growth in lean, control and obese tumor-bearing mice were assessed using a two-way ANOVA. Survival of lean, control, and obese mice tumor-bearing mice were analyzed with Kaplan-Meier curves with subsequent log rank test. Immune cell accumulation and MDSC accumulation were assessed using a one-way ANOVA, followed by Dunn's or Bonferroni correction for multiple comparisons where appropriate. For gene expression a fold change of greater than three was considered significant. All analyses were conducted using GraphPad Prism 5 software (GraphPad Software; La Jolla, CA) and statistical significance was accepted at the $p \leq 0.05$ level.

4.4. Results

4.4.1. Obesity augmented tumor growth and reduced survival in Panc.02 tumor bearing mice

Body weight (Figure 4.1a; n=8/group, two-way ANOVA, $F_{(2, 336)}=372.71$, $p \leq 0.001$) were significantly increased in obese Panc.02 tumor-bearing mice in comparison to lean and control mice. Food intake in kcal/day (Figure 4.1b; n=8/group, two-way ANOVA, $F_{(2, 320)}=425.26$, $p \leq 0.001$) were significantly increased in obese Panc.02 tumor-bearing mice in comparison to lean and control mice at all days expect at weeks 4,5, and 6, where the food intake for

obese mice was only significantly different than lean animals. Body weights and food intake were also significantly different among all dietary groups (Figure 4.1a&b; n=8/group, Bonferroni multiple comparison, $p<0.050$). Obesity increased tumor growth (Figure 4.1c; n=8/group, two-way ANOVA, $F_{(2,336)}=86.43$, $p\leq0.001$) in comparison to lean and control mice. Control mice had greater tumor growth than lean mice (Figure 4.1c; n=8/group, Bonferroni multiple comparison, $p<0.050$). Obesity significantly reduced survival in comparison to lean and control animals, and control mice had reduced survival in comparison to lean mice (Figure 4.1d; n=12/group, Log rank test, $p<0.010$).

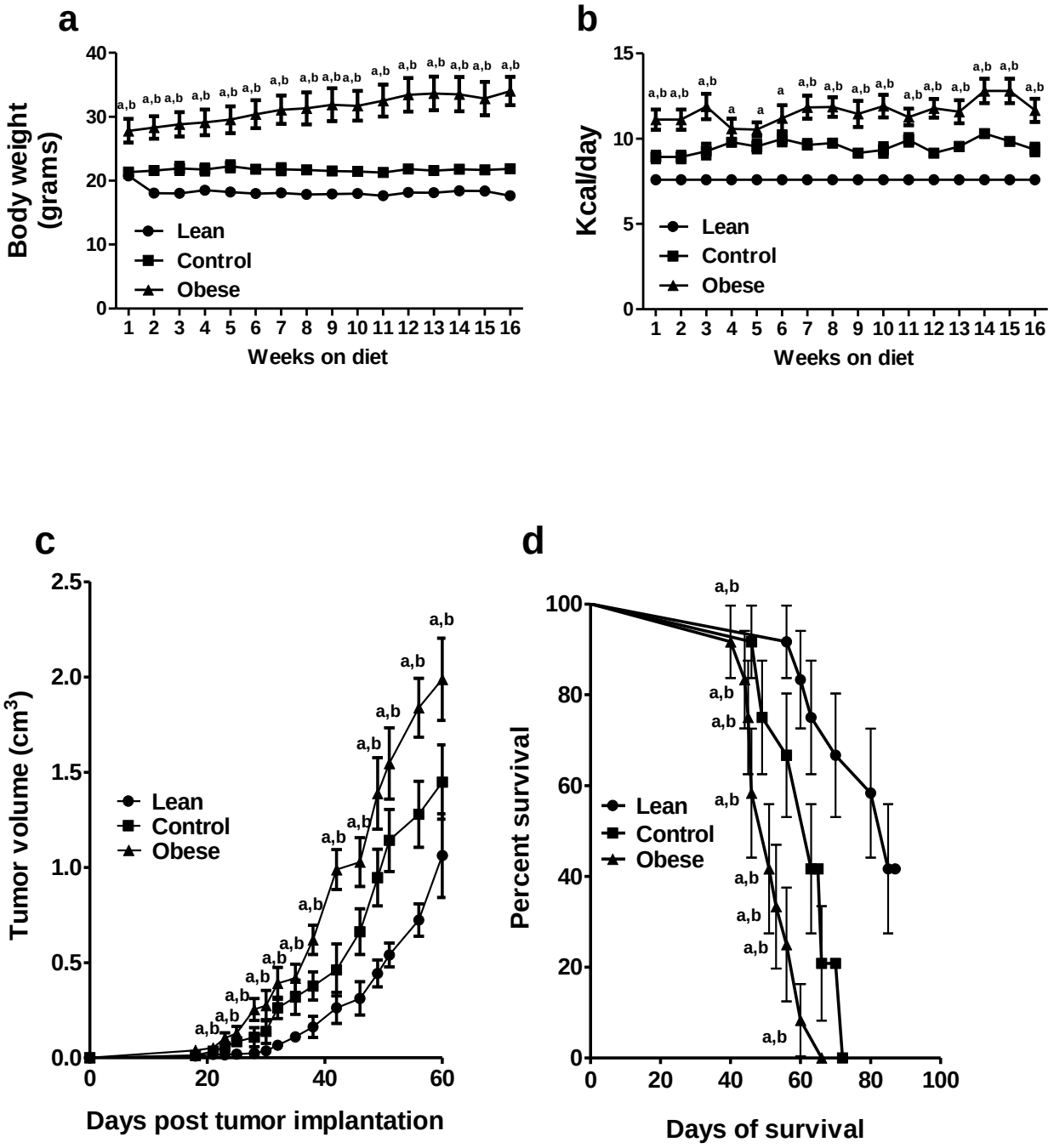

Fig. 4.1. Obesity significantly enhances body weight, food intake and tumor growth while decreasing survival in mice with Panc.02 tumors. C57BL/6 animals (n=8/group) were maintained on a 30% kcal carbohydrate calorie restricted diet, a 10% kcal diet, or 60% kcal high fat diet fed *ad libitum* to generate lean, control and obese phenotypes. After 16 weeks on the diets, Panc.02 pancreatic tumor cells ($1x10^6$) were implanted s.c. on day 0. (a) Body

weights were significantly elevated in obese tumor bearing mice in comparison to lean and control groups (a; n=8/group, two-way ANOVA, $F_{(2, 336)}$=372.71, p≤0.001). (b) Food intake (kcal/day) were significantly elevated in obese tumor bearing mice in comparison to lean and control groups at all weeks except 4,5,and 6 where the food intake for obese mice was only significantly different than lean mice (b; n=8/group, two-way ANOVA, $F_{(2, 320)}$=425.26, p≤0.001). (c) Tumor volume was significantly elevated in obese tumor bearing in mice in comparison to lean and control animals (c; n=8/group, two-way ANOVA, $F_{(2,336)}$=86.43, p≤0.001) (D). Survival curves are significantly different among lean, control and obese mice (d, n=12/group, Log rank test, p<0.010). a indicates a significant difference from lean animals. b indicates a significant difference from control animals.

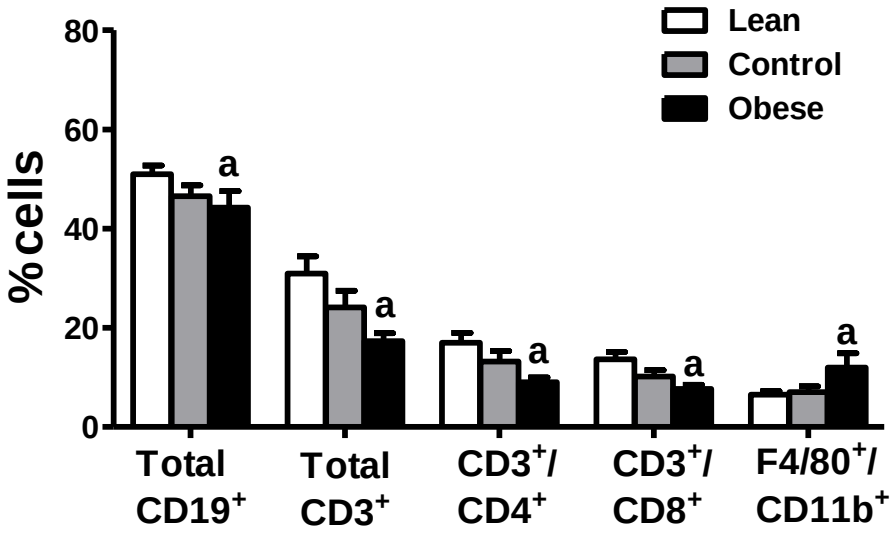

Fig. 4.2. Immune cell distribution in the spleens of lean, control and obese Panc.02 tumor bearing animals. Flow cytometric analysis was performed on splenocytes in lean, control, and obese mice (n=7-8/group) at day 60-post tumor implantation. The percentage of immune cells in the spleen, including B-cells, T cells, helper T cells, cytotoxic T cells and macrophages were quantified via flow cytomery. The percentage of total B cells (Kruskal-Wallis test; κ=6.31, p=0.043), total T cells (one-way ANOVA; p=0.016), helper T cells (Kruskal-Wallis test; κ=7.13, p=0.028) cytotoxic T cells (one-way ANOVA; p=0.008) were significantly lower in obese animals than lean animals. Macrophages were (one-way ANOVA; p=0.080, Bonferroni multiple comparison test, p<0.050) were significantly higher in obese animals than lean animals. a indicates a significant difference from lean animals.

4.4.2. Obesity altered the distribution of immune cells in the spleen of Panc.02 tumor bearing animals

Obesity significantly decreased the percentage of total B cells (Figure 4.2; n=7-8/group, Kruskal-Wallis test, κ=6.31, p=0.043), total T cells (Figure 4.2; n=7-8/group, 1-way ANOVA, p=0.016), helper T cells (Figure 4.2; n=7-8/group, Kruskal-Wallis test, κ=7.13, p=0.028) cytotoxic T cells (Figure 4.2; n=7-8/group, one-way ANOVA; p=0.008) and increased the percentage macrophages in the spleen (Figure 4.2; n=7-8/group, 1-way ANOVA, p=0.080). Bonferroni post hoc test revealed a significant increase in macrophages between obese and lean mice (Kruskal-Wallis; p<0.050). Significant differences in the percentage of immune cells in the spleen among lean and control mice, or control and obese mice were observed.

4.4.3. Obesity increased the accumulation of total (Gr-1^{+}CD11b^{+}), granulocytic (Gr-1^{Hi}CD11b^{+}) and monocytic (Gr-1^{Lo}CD11b^{+}) MDSCs in lymphoid organs of Panc.02 tumor bearing animals

A representative bivariate plot of MDSC subsets using Gr-1^{hi}/Gr-1^{low} vs. CD11b expression (Figure 4.3) is shown in the spleen of a lean, control, and obese Panc.02 tumor-bearing mouse. Distribution of MDSC subsets Gr-1^{hi}CD11b^{+} and Gr-1^{lo}CD11b^{+} among our dietary groups respectively, lean animals (2.73% and 1.90%), control animals (7.15% and 5.73%) and obese animals (13.0%, 9.14%) in a representative plot. The percentage of total MDSCs was significantly greater in the spleen (Figure 4.3b; n=7-8/group, one-way ANOVA, F=4.44, p=0.026) in comparison to lean mice. Obesity also increased

the accumulation of Gr-1^{Hi}CD11b^{+} (Figure 4.3b; 7-8/group, Kruskal-Wallis test, κ=6.43, p=0.040) and Gr-1^{Lo}CD11b^{+} (Figure 4.3b; 7-8/group, one-way ANOVA, p=0.020) MDSC subsets in the spleen in comparison to lean mice. Obese and control mice accumulated significantly more total MDSCs in the TDLN (Figure 4.3c; n=8/group, one-way ANOVA, p<0.050) in comparison to lean Panc.02 tumor bearing mice. Also in the TDLN, we observed a trend towards more MDSC subsets in obese mice; however this did not reach statistical significance. Obese mice accumulated more total MDSCs in the tumor (Figure 4.3d; n=2-5/group, Kruskal-Wallis test, κ=5.15, p=0.060). Dunn's post hoc test showed a significant difference between MDSCs in the tumor of obese and lean mice (d, Kruskal-Wallis; p<0.050). Gr-1^{Lo}CD11b^{+} (Figure 4.3d; 2-5/group, Kruskal-Wallis test, κ=5.51, p=0.043) MDSCs were significantly elevated in the tumors of obese mice in comparison to lean mice. No differences were observed between the percentage of total, Gr-1^{Hi}CD11b^{+}, and Gr-1^{Lo}CD11b^{+} MDSC subsets from lean and control mice and control and obese mice.

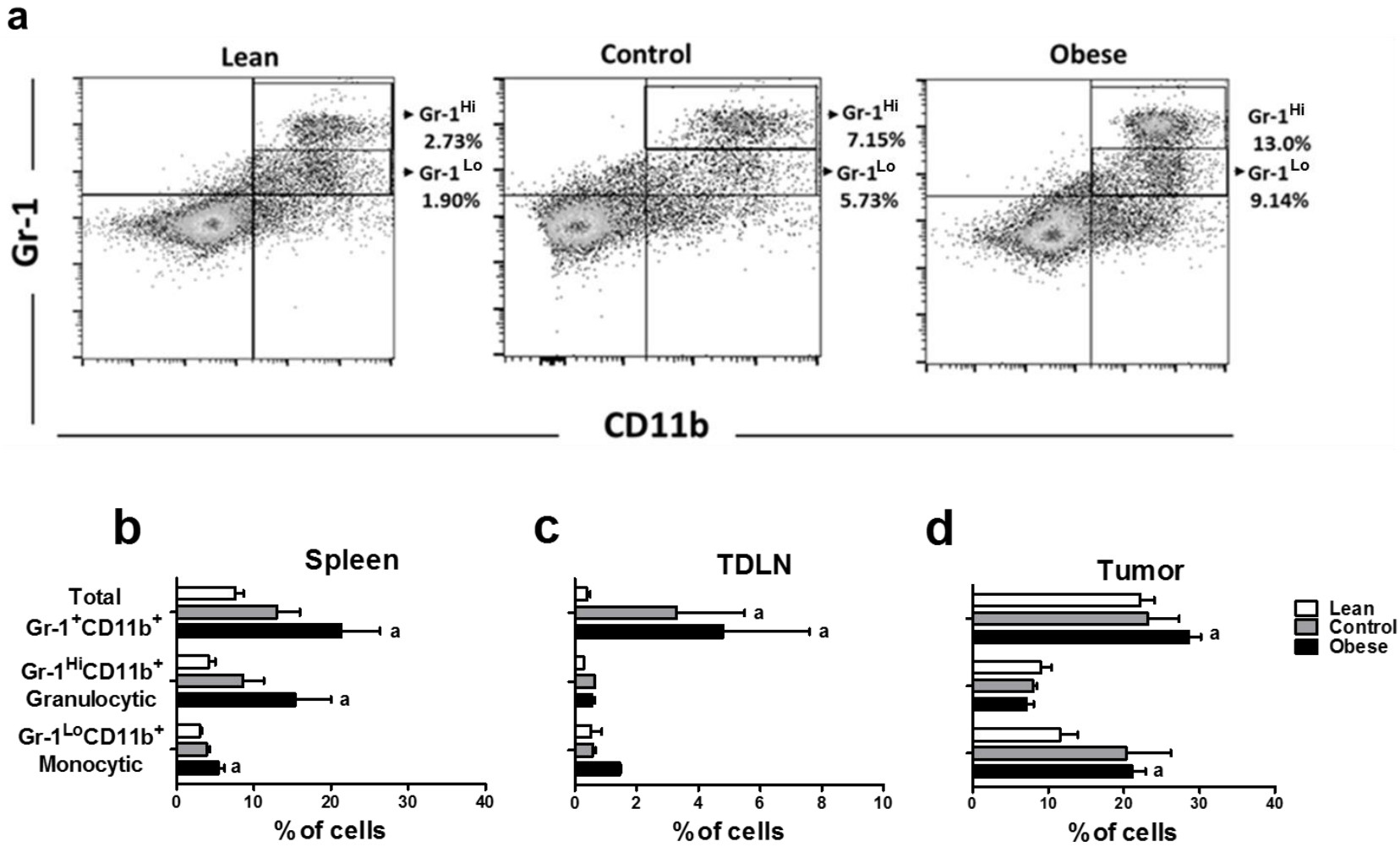
a
Lean
Control
Obese
Gr-1
CD11b
Gr-1Hi
2.73%
Gr-1Lo
1.90%
Gr-1Hi
7.15%
Gr-1Lo
5.73%
Gr-1Hi
13.0%
Gr-1Lo
9.14%
b
Spleen
Total
Gr-1+CD11b+
Gr-1HiCD11b+
Granulocytic
Gr-1LoCD11b+
Monocytic
a
0
10
20
30
40
% of cells
c
TDLN
a
0
2
4
6
8
10
% of cells
d
Tumor
a
0
10
20
30
40
% of cells
Lean
Control
Obese

Fig. 4.3. Accumulation of Gr-1^{+}CD11b^{+} cells in lean, control, and obese Panc.02 tumor bearing animals. Flow cytometric analysis was performed on spleen, TDLN and tumors at day 60 post-tumor implantation. (a) Representative bivariate plot using Gr-1^{hi} /Gr-1^{low} vs. CD11b expression is shown in the spleen of a lean, control, and obese Panc.02 tumor-bearing mouse day 60 post-tumor implantation. Arrows indicate different subpopulations of MDSCs subsets (Gr-1^{Hi}CD11b^{+} and Gr-1^{Lo}CD11b^{+}) in lean (2.71%, 1.90%), control (7.15% and 5.73%) and obese (13.0% and 9.14%) Panc.02 tumor-bearing animals, respectively. The percentage of total, Gr-1^{Hi}CD11b^{+}, and Gr-1^{Lo}CD11b^{+} MDSCs is displayed in the (b) spleen, (c) TDLN, and (d) tumor of lean, control, and obese Panc.02 tumor-bearing animals at day 60-post tumor implantation. (b) The percentage of MDSCs was significantly higher in obese animals than lean animals at day 60 post tumor implantation in the spleen (b, n=7-8/group, one-way ANOVA; F=4.44, p=0.026). The percentage of Gr-1^{Hi}CD11b^{+} (b, n=7-8/group, Kruskal-Wallis test; κ=6.43, p=0.040) and Gr-1^{Lo}CD11b^{+} (b, n=7-8/group, one-way ANOVA, p=0.020) MDSCs in the spleen were significantly elevated in obese mice in comparison to lean mice. No differences were observed in the spleen between total, Gr-1^{Hi}CD11b^{+}, and Gr-1^{Lo}CD11b^{+} MDSC subsets from lean and control mice and control and obese mice. (c) Obese and control mice accumulated significantly more total MDSCs in the TDLN (c, n=8/group, one-way ANOVA; p<0.050) in comparison to lean mice. No significant differences were observed among MDSC subsets in TDLN among dietary groups. (d) Obese mice accumulated more total MDSCs in the tumor in comparison to lean mice (d; n=2-5/group, Kruskal-Wallis test; κ=5.15, p=0.060). Dunns post hoc test showed a significant difference between total MDSCs in the tumor of obese mice in comparison to lean mice (d, Kruskal-Wallis, p<0.050). Obese mice accumulated more Gr-1^{Lo}CD11b^{+}MDSC in the tumor (d; n=2-5/group, Kruskal-Wallis test; κ=5.51, p=0.043) in comparison to lean mice. No differences were observed in the tumor between total, Gr-1^{Hi}CD11b^{+}, and Gr-1^{Lo}CD11b^{+} MDSC subsets from lean and control mice and control and obese mice. a indicates a significant difference from lean animals.

4.4.4. Obesity altered adipocyte size in Panc.02 tumor-bearing mice

Adipose tissues from a lean, control and obese Panc.02 tumor-bearing animal were stained with H & E for histological examination (Figure 4.4a). Obese mice had larger adipocytes in comparison to lean and control mice. Adipocytes from lean and control mice were similar in size. Immunofluorescence of a representative lean, control and obese mouse is shown (Figure 4.4b). Adipose

tissue was stained with antibodies to Gr-1 (red) and CD11b (green). Lean, control, and obese tumor-bearing mice accumulated MDSCs in adipose tissue.

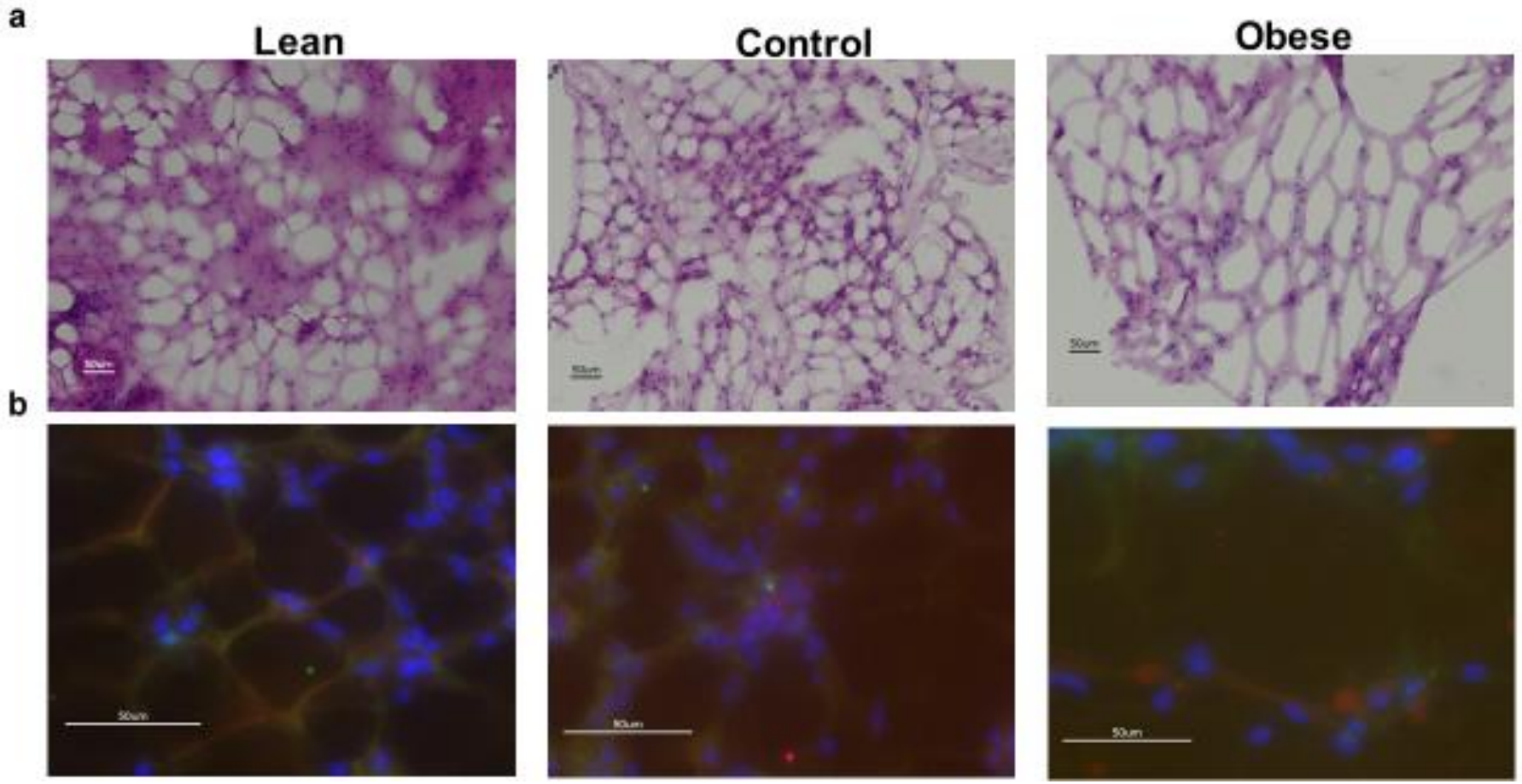
a
Lean
Control
Obese
b

Fig. 4.4. Increased expression of Gr-1 and CD11b in the adipose of obese Panc.02 tumor-bearing mice. Photomicrographs are representative of fields. (a) H&E stained tissue sections of adipose tissue from lean, control and obese mice. Magnification 20X. (b) Adipose tissue from lean, control and obese mice was stained with antibodies to Gr-1 (red) and CD11b (green) and DAPI (blue) was used for the nuclear stain. Magnification 80X.

4.5.5. Obesity increased gene expression of Gr-1 and inflammatory mediators in adipose tissue

Gene expression of Gr-1, ARG-1, iNOS, TGF-β, CD3, IL-6, IL-10, and Cox-2 were measured in adipose tissue isolated from lean, control and obese Panc.02 tumor bearing animals (Figure 4.5; n=3-5/group) by RT-PCR. Obesity increased gene expression of Gr-1 in comparison to lean and control mice (Figure 4.5a). Gene expression of IL-6, IL-10, and Cox-2 were elevated in obese mice in comparison to lean mice (Figure 4.5b). Cox-2 was also elevated in control mice in comparison to lean and obese mice. We did not observe any differences between lean and control mice for the adipose tissue gene expression of CD3, IL-6, IL-10, Gr-1, ARG-1, iNOS, and TGF-β. Also, we did not observe any differences between control and obese mice for the adipose tissue gene expression of CD3, ARG-1, iNOS, and TGF-β.

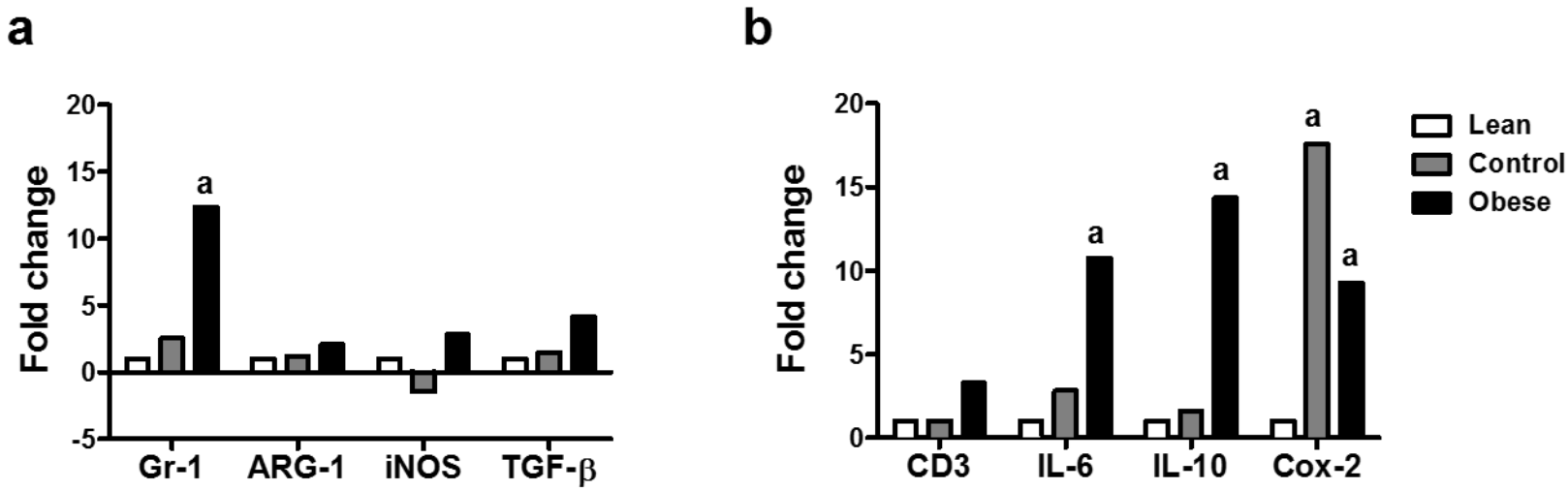

Fig. 4.5. Accumulation of MDSCs subsets lean, control, and obese Panc.02 tumor bearing animals. Gene expression of (a) Gr-1, ARG-1, iNOS, TGF-β and (b) CD3, IL-6, IL-10, Cox-2 and were measured in adipose tissue isolated from lean (n=6), control (n=5) and obese (n=3) Panc.02 tumor bearing animals by RT-PCR. Expression levels were normalized to 18s RNA and fold change was determined using the $2^{\Delta\Delta CT}$ method. Fold change greater than 3 was considered significant. Expression levels of IL-6, IL-10, Cox-2, Gr-1, and TGF-β were significantly elevated in obese mice in comparison to lean mice. Cox-2 was elevated in control mice in comparison to lean and obese mice. No differences were observed between lean and control mice and control and obese mice for the adipose tissue gene expression of CD3, ARG-1, iNOS, and TGF-β. a indicates a difference from lean animals. b indicates a difference from control animals. c indicates a difference from obese animals.

4.6.6. Splenic MDSCs from lean, control, and obese Panc.02 tumor bearing mice suppressed T cell proliferation

To test the ability of MDSC subsets from lean, control, and obese tumor-bearing mice to inhibit T cell proliferation, T cells from BALB/c mice were cultured with APCs from Panc.02 tumor bearing mice in a mixed lymphocyte reaction (MLR) with and without MDSC subsets from Panc.02 tumor bearing mice in all dietary groups. No differences were observed in the suppressive capacity of MDSC subsets from lean, control, and obese mice (Figure 4.6).

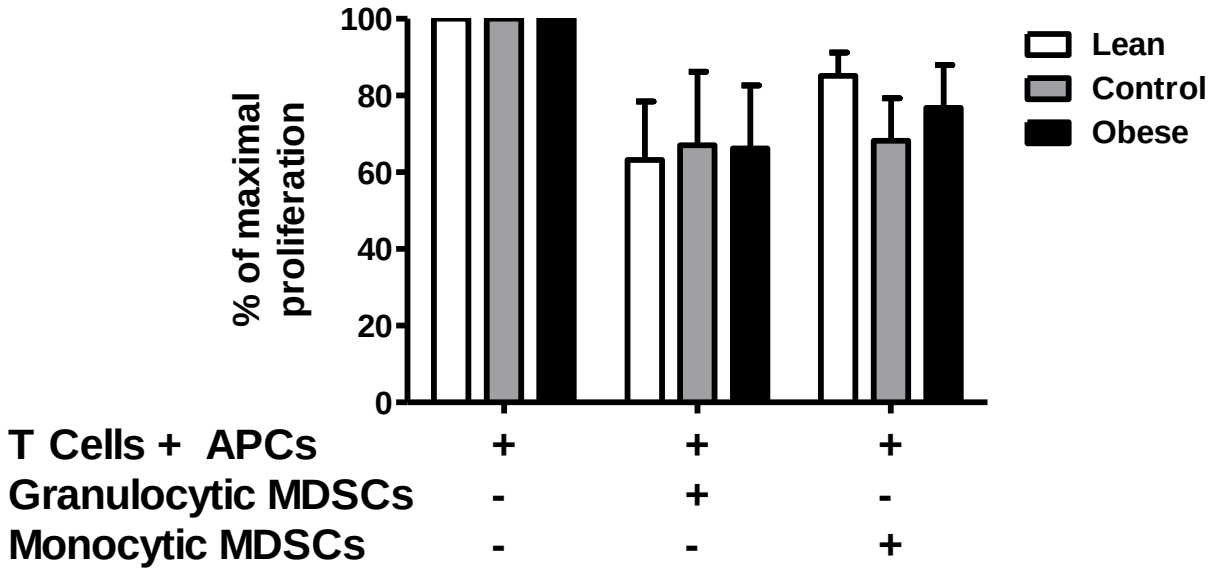

Fig. 4.6. Suppression of T cell proliferation by MDSCs from lean, control, and obese Panc.02 tumor bearing animals. Irradiated APCs (2000 Rads) and MDSC subsets were isolated from lean, control, and obese Panc.02 tumor bearing animals (n=4-8/group) day 60 post-tumor implantation and mixed with T cells isolated from non-tumor bearing BALB/c mice. Cells were incubated for 5 days and proliferation was measured via thymidine incorporation. Percent of maximal proliferation (i.e. proliferation of T cell plus APCs alone) is reported for

animals with tumor volumes larger than 1cm^3. MDSCs subsets alone did not proliferate (data not shown). MDSC subsets from mice among all dietary groups suppressed T cell proliferation to the same extent.

4.7.7. Obesity drives the accumulation of Gr-1$^+$CD11b$^+$ cells in the spleen of non-tumor bearing animals

Lean, control, and obese non-tumor bearing mice accumulate MDSCs in the spleen in the absence of Panc.02 tumor and tumor-derived factors. A trend towards more MDSCs in obese mice was observed, however this did not reach statistical significance (Figure 4.7; n=3/group, Kruskal-Wallis test, κ=2.75, p=0.252).

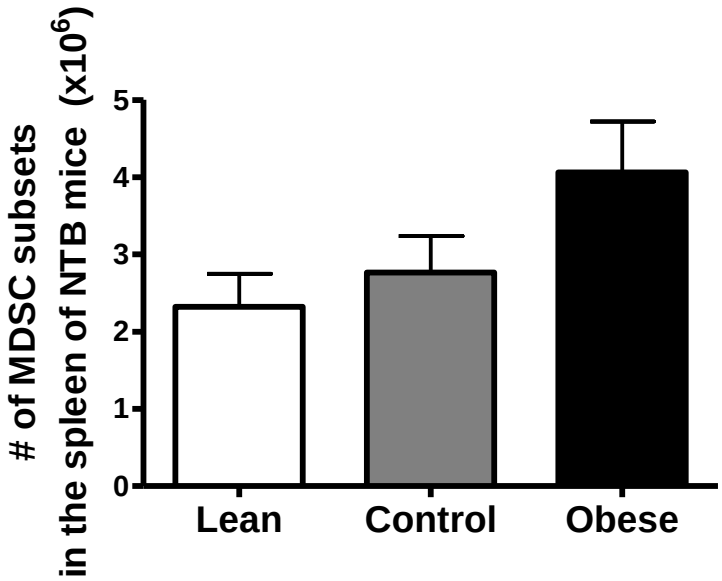

Fig. 4.7. Accumulation of Gr-1$^+$CD11b$^+$ cells in the spleen of non-tumor bearing lean, control, and obese animals. Flow cytometric analysis was performed on splenocytes in non-tumor-bearing lean, control, and obese mice. The number of Gr-1$^+$ vs. CD11b$^+$ cells in the spleen of non-tumor-bearing lean, control, and obese mice. A trend towards more MDSCs in obese mice was observed, however it did not reach statistical significance (n=3/group, Kruskal-Wallis test, κ=2.75, p=0.252). Test for trend p=0.059.

4.8. Inflammatory mediators in serum lean, control, and obese TF mice.

Inflammatory mediators were quantified in the plasma of lean, control, and obese TF (n=3-5/group) mice before tumor injection. The percentage on animals above the detection limit is shown. No significant differences were observed between levels of inflammatory mediators and growth factors among dietary groups. A trend toward more IL-6, and TNF-α is observed in obese TF animals in comparison to lean and control animals.

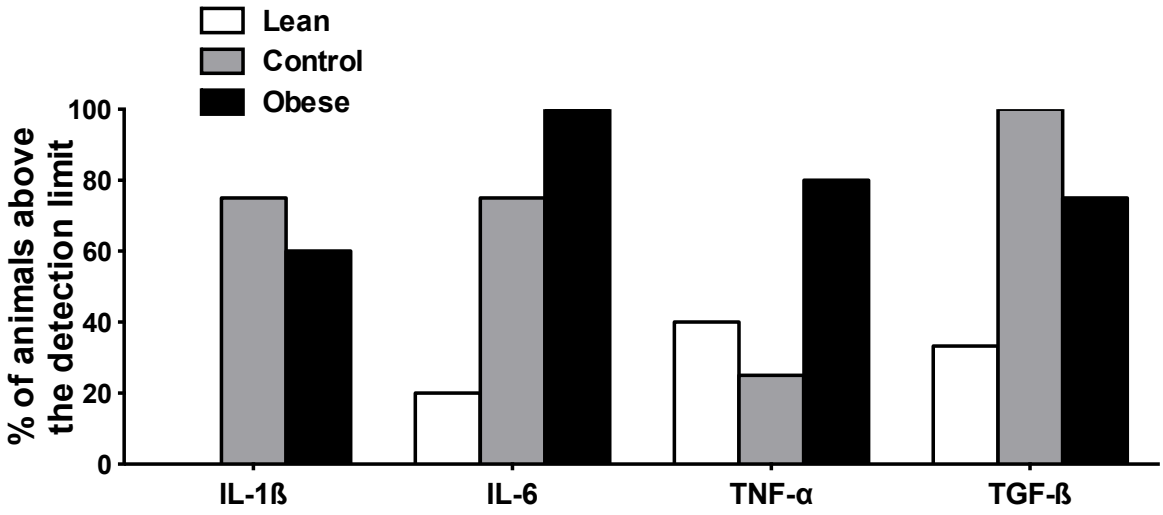

Fig. 4.8. Percentage of lean, control, and obese TF animals above the detection limit for plasma levels of IL-1β, IL-6, TNF-α and TGF-β. Plasma levels of G-CSF, GM-CSF, IL-1β, IL-6, KC, MCP-1, MIP-1α, and TNF- α were measured in lean, control and obese TF animals (n=3-5/group using the MILLIPLEX MAP Cytokine array. Plasma levels of TGF-β were measured in lean, control and obese TF animals (n=3-5/group) via ELISA.

4.5. Discussion

We demonstrated that obesity increased tumor growth and decreased survival in Panc.02 tumor bearing mice. Obesity also increased the accumulation of total MDSCs in all tissue compartments, but the tissue distribution of granulocytic and monocytic MDSC subsets varied by tissue compartment. Obesity augmented gene expression of Gr-1 and IL-6, IL-10, and Cox-2 in adipose of Panc.02 tumor-bearing mice. MDSC subsets from all dietary groups were equally suppressive, regardless of the dietary intervention. This is the first study to date to demonstrate that obesity can enhance tumor growth, decrease survival, and increase the accumulation of MDSCs in the spleen, TDLN, tumor, and adipose of Panc.02 tumor bearing mice

Obesity can increase the incidence of pancreatic cancer (43, 44, 90, 194-197). Recent epidemiology studies show obesity is associated with a reduction in survival from pancreatic cancer (92, 101, 187). Similar studies have been performed in pre-clinical models of obesity and pancreatic cancer. Diet induced obesity (DIO) increases tumor volume, blood glucose, insulin, leptin, IGF-1, and reduces levels of adiponectin when compared to calorie-restricted mice (96, 108). The progression from intraepithelial lesions to pancreatic ductal adenocarcinoma (PDAC) is delayed in calorie-restricted mice (198) in both orthotopic (NB508 cancer cells derived Kras/Ink4a genetic model of pancreatic ductal adenocarcinoma) and genetic models (Kras/Ink4a$^{+/-}$) of pancreatic cancer. Obesity enhances pancreatic tumor growth, increases metastasis, and alters serum levels of leptin, insulin and glucose (96) in a subcutaneous model of

pancreatic cancer in mice deficient for both leptin and its receptor. Larger tumors and less tumor cell apoptosis are present in overweight mice in comparison to lean mice, in a subcutaneous model of pancreatic cancer in DIO model (108). These studies utilizing murine models of pancreatic cancer in both genetic and diet-induced models of obesity demonstrate that obesity enhances pancreatic tumor growth, increases metastasis, and alters serum levels of adipokines. Our data demonstrate that obese mice had a larger tumors and a significant reduction in survival in comparison to lean and control mice; thus our results are consistent with the previous literature assessing the link between obesity and pancreatic (108). These data suggest that lean, calorie restricted mice have reduced tumor growth, and better survival outcomes, while obese mice have enhanced tumor growth and poorer survival outcomes independent of the paradigm used to generate obesity or the model of pancreatic cancer. These data in murine models of pancreatic cancer are consistent with the epidemiological data assessing the link between obesity and pancreatic risk and mortality (101, 194).

Previous studies have shown that obesity can modulate immune function (46). Our data demonstrate that obesity decreased the percentage of B cells and $CD4^+$ and $CD8^+$ T cells, while obesity increased the percentage of macrophages in the spleen. This data demonstrates that obesity can alter the percentage of immune cells in lymphoid organs. Clinical studies have also shown that obesity can alter the number of immune cells in peripheral blood, including T cells, NK cells, and B cells (46).

In addition to obesity-induced impairments in anti-tumor immunity, obesity may be increasing tumor-derived immunosuppressive factors including MDSCs. MDSCs are hematopoietic progenitor cells and precursors of macrophages, dendritic cells (DCs) and granulocytes. MDSCs are a potent immunosuppressive cell population with the ability to suppress both innate and adaptive immune response (171). Thus they contribute to disease progression by impairing host antitumor immunity. MDSCs accumulate in pancreatic cancer patients, and their accumulation correlates with disease progression (27, 182). The accumulation of MDSCs in cancer patients is also associated with a concurrent reduction in $CD8^+$T cells (28). These results demonstrate that MDSCs mediate immune suppression in pancreatic cancer patients.

Similarly, MDSCs are documented in murine models of pancreatic cancer including transgenic, orthotopic (199), and subcutaneous (38) models; and the accumulation of MDSCs is organ dependent (183). We demonstrated that obesity increased the accumulation of total MDSCs in all tissue compartments. In the current study, obesity significantly increased the accumulation of MDSCs in the spleen, TDLN, tumor, and adipose in comparison to lean mice. Control mice also accumulated more MDSCs in the TDLN in comparison to lean mice. No significant differences in MDSC accumulation in the spleen, TDLN, tumor and were observed between lean and control mice or between obese mice and control mice. These data suggests that the maintenance of body weight over time, as observed in the lean mice, may have a protective effect. This data also imply that obesity contributes to immune suppression in pancreatic cancer by

altering the accumulation of MDSCs. An increased number of MDSCs in obese animals may provide a robust inhibitory signal to prevent immune recognition of tumor antigens; thus, the obesity-induced accumulation of MDSCs may contribute significantly to the obesity-induced exacerbation of tumor development.

Murine MDSCs are comprised of two subsets, including a Gr-1^{Hi}CD11b^{+} (granulocytic) and a Gr-1^{Lo}CD11b^{+} (monocytic) subset. In this study, we demonstrate that obesity significantly increased the expansion of both the granulocytic and monocytic MDSC subsets in the spleen. A similar trend was also observed in the TDLN, but it did not reach statistical significance. We also demonstrate that obesity significantly increased the monocytic MDSC subset in the tumor of Panc.02 tumor-bearing mice in comparison to lean mice. We did not observe any significant differences in MDSC subset accumulation between lean and control animals or control and obese animals. However, a trend towards a stepwise effect on MDSC accumulation in the spleen and TDLN among our dietary groups was noticed, however it did not reach statistical significance. We also noted a preferential expansion of the granulocytic MDSCs in the spleen and in the TDLN, in contrast to an accumulation of the monocytic MDSCs in the tumor. This suggests that obesity can alter MDSC subset distribution, in a tissue specific manner. MDSC subsets expand in lung, melanoma, colon, and breast tumor models (146). 70-80% of the MDSC expansion in lymphoid organs and tumor is attributed to accumulation of the granulocytic subset and 20-30% is due to the monocytic subset (146) in the aforementioned tumor models. In the

peripheral blood of pancreatic cancer patients granulocytic MDSCs are more prevalent in comparison to monocytic MDSC subset (182). The accumulation of MDSCs observed in this study is consistent with other studies that document MDSCs accumulation in murine models of pancreatic cancer (171, 199, 200). The data presented is the first to demonstrate an obesity-driven expansion of two distinct MDSC subsets in a murine model of pancreatic cancer. These data suggest that obesity contributes to immune suppression in pancreatic cancer by altering the expansion of MDSC subsets.

Due to the immunosuppressive nature of MDSCs in pancreatic cancer, preclinical studies are currently exploring ways to exploit their accumulation and function. Gemcitabine in combination with a dendritic cell vaccination induces immunity in the mice with pancreatic cancer (145). Stromnes *et al.* utilized a genetically engineered mouse model of PDAC that progress with disease similar to humans. The authors found that antibody depletion of the granulocytic MDSC subset reduces the accumulation of MDSCs in the peripheral blood; spleen and tumor. More importantly this study found that depletion of the granulocytic MDSC subset increases the accumulation of $CD8^+$ T cells in the tumor and induces apoptosis of tumor cells (171). Collectively these data demonstrate that MDSCs suppress anti-tumor immunity and depletion of MDSCs in pancreatic tumor models can enhance antitumor immunity. These studies also provide a direct link for MDSCs in increasing tumor burden, via the expansion of MDSCs in models of pancreatic cancer.

MDSCs are known to suppress innate and adaptive immunity through a variety of mechanisms (12, 201). MDSCs can also regulate innate immune responses by modulating the cytokine production of macrophages, by skewing macrophages toward a tumor-promoting phenotype via the production of IL-10 (21). We observed increased gene expression of IL-6, IL-10, and Cox-2 in the adipose of obese mice in comparison to lean mice. Cox-2 expression was greater in the adipose of control mice in comparison to lean mice. No differences in gene expression of CD3, ARG-1, iNOS and TGF-β were observed between lean and control mice or control and obese. These data suggest that obesity-induced expansion of MDSCs may be altering macrophages towards a M2 tumor promoting phenotype, which may potentiate pancreatic cancer growth. Macrophages are important in regulating immunity, and can be classified as M1 classically activated and M2 alternatively activated, however these are not exclusive (202). M2 macrophages are activated on a continuum in response to various agents. M2a (alternative) macrophages are polarized by IL-4 and IL-13, they are important in Th2 responses and inflammatory responses. Immune complexes and toll like receptor (TLR) triggering polarize M2b (type II) macrophages, and they are important for immune-regulation. Lastly, M2c (deactivated) macrophages are polarized by IL-10, TGF-β and are important in immune-regulation and matrix and tissue remodeling (203). For the purpose of this discussion, we refer to M2 macrophages, which functions include matrix remodeling, tissue repair, and angiogenesis; all of which favor tumor promotion (202). Xia and colleagues show Gr-1^{+}CD11b^{+} cells accumulate in the adipose of

obese (ob/ob) non-tumor bearing animals in comparison to WT controls and improved insulin sensitivity, reduced serum IL-6 and TNF-α and skewed macrophage polarization towards an M2 phenotype; suggesting that MDSCs may be negative regulators of obesity-induced inflammation in the absence of tumor (83). Our data demonstrate that in pancreatic tumor-bearing animals, obesity results in the accumulation MDSC and immunosuppressive factors in adipose tissue. It is not known if the accumulation of MDSCs observed in obese adipose tissue is to dampen the inflammatory response as observed as a result of obesity.

To separate the effects of obesity-induced inflammation from tumor-derived factors, which stimulate MDSC expansion, we examined the effect of obesity in non-tumor bearing animals on MDSC formation. We observed an increase in the number of Gr-1^{+}CD11b^{+} cells in the spleen of non-tumor bearing obese animals. These data suggest that MDSCs accumulate independent of the tumor microenvironment likely due to the pro-inflammatory state observed with obesity; suggesting a role for the adipose in driving MDSCS accumulation.

Various inflammatory and tumor derived factors can activate MDSCs, which increases the production of immune suppressive factors, including ARG-1, iNOS, ROS (12), TGF-β and IL-10 (21, 204). MDSC subsets are hypothesized to mediate their suppressive function through different mechanisms via transcription factors STAT1 and STAT3. In the current study, we demonstrate that granulocytic and monocytic MDSCs suppressed T cell proliferation regardless of the dietary intervention. We also measured gene expression of

immunosuppressive factors (IL-6, IL-10, ARG-1, iNOS, Cox-2, and TGF-β) and transcription factors (STAT1 and STAT3) in MDSC subsets from lean, control, and obese Panc.02 tumor bearing mice.

We observed a down-regulation in gene expression of IL-6, IL-10, ARG-1, iNOS, Cox-2, TGF- β, STAT1 and STAT3 measured in control and obese mice in comparison to lean mice. iNOS was down regulated in granulocytic MDSCs from obese mice to a greater extent than control mice. ARG-1 was down regulated in monocytic MDSCs from obese mice to a greater extent than control mice. These data suggest that obesity is not altering the suppressive capacity of MDSCs; however, obesity increased the number of MDSCs on a per cell basis.

Obesity is characterized as low-grade chronic inflammation, with increased inflammatory mediators including IL-1α, IL-1β, IL-6, TNF-α, and MCP-1 (57). The tumor microenvironment is also comprised of numerous cytokines (IL-6, IL-1 β, TGF-β, IL-10) (134) and growth factors (VEGF, GM-CSF, G-CSF) that can also support tumor progression (205). IL-1β, IL-6 (16, 19, 20), TGF-β (185) and the prostaglandin, PGE_2 (21) are mediators of carcinogenesis; however they can also induce MDSC accumulation. In this study we noted increased levels of IL-6, IL-1β, TNF-α, and TGF-β in obese mice in comparison to lean controls, however these relationships did not reach statistical significance. Inflammatory mediators IL-6, IL-8, and MIP-1α play a role in pancreatic carcinogenesis. IL-6 positively correlates with tumor stage as is associated with poor outcome in pancreatic cancer patients. Similarly, IL-8 is associated with angiogenesis, metastasis, and negatively influences the survival of tumor bearing mice. MIP-1α

enhanced proliferation, induced epithelial to mesenchymal transition, and is associated with overall poor prognosis. These data suggest that increased inflammatory mediators (i.e. IL-6) may contribute to the expansion of MDSCs.

In conclusion, obesity enhanced tumor growth and decreased survival concurrently with the expansion of MDSCs in lymphoid organs. These data suggest that obesity increases pancreatic carcinogenesis via the accumulation of MDSCs. Results from the current study provide critical insight into additional mechanisms linking obesity and pancreatic cancer via immunosuppression by MDSCs. These results contribute to our understanding of obesity on inflammatory and tumor-associated immunosuppressive factors.

Supplementary Table 2. Fold change in gene expression of and transcription factors immunosuppressive factors, immune cells in tumors from lean, control and obese Panc.02 tumor-bearing mice.

Supplementary Table 2. Fold change in gene expression of transcription factors, and immunosuppressive factors in tumor of Panc.02 mice		
	Control	**Obese**
ARG-1	-1.07	-1.29
iNOS	-1.14	1.05
Cox-2	1.1	-1.49
TGF-β	1.58	-1.13
IL-6	-1.7	-3.98
IL-10	-1.17	-1.16
STAT1	-1.1	-1.1
STAT3	2.09	1.39
CD3	-1.49	-1.52

Supplementary Table 3. Fold change in gene expression of transcription factors immunosuppressive factors in MDSC subsets from lean, control and obese Panc.02 tumor-bearing mice.

Supplementary Table 3. Fold change in gene expression of transcription factors, and immunosuppressive factors in MDSC subsets				
	Granulocytic		Monocytic	
	Control	Obese	Control	Obese
ARG-1	-1.43	-1.96	-1.42	-18.63
iNOS	-2.01	-5.48	-5.77	-5.62
Cox-2	-3.79	-3.06	-3.32	-1.34
TGF-B	-3.31	-3.75	-1.62	-2.4
IL-6	-17.41	-1.75	-1.16	-1.56
IL-10	-2.62	-2.22	-1.53	-4.51
STAT1	-3.1	-2.87	-2.3	-0.33
STAT3	-1.5	-1.99	-1.36	-1.86

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The studies reported in this dissertation were designed to explore MDSC and MDSC subset accumulation and function, and their contribution to tumor growth in a murine model of pancreatic cancer. These studies also were designed to investigate the inflammatory milieu in this model. Additionally, the effect of obesity on pancreatic tumor growth, survival, and MDSC an MDSC subset accumulation and function were explored. This chapter briefly summarizes the main findings and identifies future directions for research.

Study 1 characterized the inflammatory milieu, tumor microenvironment, MDSC accumulation, an MDSC subset accumulation and function in TF and Panc.02 tumor bearing animals at days 30-60 post tumor implantation. The characterization of MDSCs is an important contribution to the field because they are potent inhibitors of anti-tumor immune mechanisms, and they are correlated with disease stage and severity in pancreatic cancer patients. Understanding the biology of MDSCs in pre-clinical models of pancreatic cancer is critical to understanding how these contribute to anti-tumor immunity in this Pan.02 tumor model. Pancreatic cancer is one of the most deadly cancers, and is associated with a very poor survival rate. The five-year survival rates for pancreatic cancer in 1975 were three percent and the current survival rate is approximately five percent (206). Unfortunately clinically relevant advances in treatment for pancreatic cancer are lacking. Pancreatic carcinogenesis is a complex stepwise disease progression comprised of genetic alterations (e.g. activating mutations in

KRAS, and inactivating mutations in p53, and SMAD4), inflammatory modulations (e.g. increases in pro-inflammatory mediators IL-6, IL-8, TNF-α, IL-β and TGF-β), and shifts in immune cell populations (e.g. reduction in $CD8^+$ T cells, increases in Tregs and MDSCs). Increases in levels of Tregs and MDSCs in pancreatic cancer patients, which correlate with overall poor prognosis, suggest a role for these immunosuppressive cells in the potentiation of tumor growth.

It is also known that MDSC accumulation is tumor model dependent. The accumulation of MDSCs can be driven by various growth factors and inflammatory mediators (e.g. IL-6, GM-CSF, VEGF and PGE_2); and since the inflammatory milieu varies by tumor model, it likely effects/influences MDSC accumulation differentially. Thus, a greater understanding the biology of MDSCs in pre-clinical models of pancreatic cancer is critical for development future immunotherapies. In this study we demonstrated that Panc.02 tumor bearing mice have higher levels of the growth factors GM-CSF and VEGF, and inflammatory mediators IL-6, TNF-α, MCP-1, MIP-1α, and TGF-β in comparison to TF mice. These results confirm that the Panc.02 model is conducive for the expansion of MDSCs.

We also found that MDSCs accumulated overtime, with a greater expansion of the granulocytic MDSC subset. MDSCs are comprised of a mixed population of immature granulocytes, macrophages and dendritic cells, thus the characterization of MDSCs includes cell surface markers for immature myeloid cells. Due to the heterogeneous nature of MDSCs, inconsistencies exist in the literature with regards to the terminology of MDSCs. We show for the first time

that accumulation of MDSCs was evident and comparable using either the Gr-1/CD11b or the CD11b/ Ly6C/Ly6G nomenclature. This data are important as it confirms we are adequately capturing the MDSCs cell population.

MDSCs can inhibit the function of immune cells including T cells, DCs and NK cells. We also found that MDSC subsets from Panc.02 tumor bearing mice suppressed T cell function to a similar extent. Upon further examination of the suppressive capacity of MDSC subsets, we found that both MDSCs subsets expressed immunosuppressive factors iNOS, and IL-6 and transcription factors STAT1 and STAT3, however only the monocytic subset expressed IL-10 and ARG-1 and only the granulocytic subset expressed TGF-β. It is suggested that MDSC subsets mediate their suppressive effects through STAT1 and STAT3 differentially (12). However, we noted similarities in the amount of STAT1 and STAT3 in both MDSC subsets. To further examine the production of immunosuppressive factors TGF-β, IL-6 and IL-10, we measured protein expression. TGF-β, IL-6 and IL-10 were produced in both MDSCs subsets, but levels were not significantly different among MDSC subsets. These results demonstrate that MDSC subsets are functional and have the capacity to suppress T cell function, likely through production of TGF-β and IL-10.

Next we wanted to determine the overall contribution of MDSCs to Panc.02 tumor growth in this murine pancreatic tumor model. We found that depletion of MDSCs reduced tumor growth. These results suggest that in this Panc.02 tumor model, tumor growth is driven and exacerbated by MDSCs. Our results also suggest that elevated inflammatory mediators are responsible for the

greater accumulation of MDSCs in Panc.02 tumor bearing mice. Production of TGF-β and IL-10 in both MDSC subsets may independently or collectively be responsive for the reduction in T cell proliferation we observed. TGF-β (207) and IL-10 (208) can inhibit the function of immune cells including T cells, NK cells, neutrophils and macrophages. It is likely that both TGF-β and IL-10 work collectively to reduce the T cell proliferation that we observed in our study. This study has enabled us to better understand the inflammatory environment, the role of MDSCs, and MDSC subset accumulation and function, and their overall contribution to tumor growth in Panc.02 tumor model.

Future studies should determine if the reduction in T cell function we observed in MDSC subsets in directly attributed to the production of immunosuppressive cytokines TGF-β and IL-10. Inhibitors to TGF-β and IL-10 can be added to MLR cultures including T cells, APCs and MDSCs subsets to determine the contribution of TGF-β and IL-10 to T cell proliferation.

Also, we observed increased levels of IL-6 and GM-CSF in plasma of Panc.02 tumor-bearing mice, which are known to drive the expansion of MDSCs (12, 19). Future studies could determine if IL-6 and GM-CSF drive accumulation of MDSCs in the Pacn.02 tumor model. Bone marrow cells from Panc.02 tumor bearing mice and from control mice, can be treated with IL-6 and GM-CSF *in vitro* to see if IL-6 and GM-CSF drive expansion of MDSCs. Similarly, IL-6 and GM-CSF knock out (KO) mice and wild type (WT) controls can be used to determine MDSCs accumulation in murine model of pancreatic cancer. Panc.02

cells could be injected s.c. into KO mice and WT controls and the accumulation of MDSCs in lymphoid organs and tumors could be determined.

It is not known if combination of Gr-1 depletion antibody and neutralizing antibody to GM-CSF would offer greater effects on reduction of tumor growth than just depletion antibody alone. Panc.02 tumor bearing mice could be treated with both Gr-1 depletion antibody and neutralizing antibody to GM-CSF. End point analysis could determine if the combination of Gr-1 and GM-CSF depletion antibodies alters tumor growth and survival characteristics. The clinical impact of combined chemotherapy and immunotherapy in pancreatic cancer patients is currently under investigation. As MDSCs are such potent inhibitors of anti-tumor immunity, combination treatment might offer promising avenues in pancreatic cancer patients.

Study 2 tested the hypothesis that obesity induces an increase in immunosuppressive factors, which enhances pancreatic tumor growth. It is known that obesity increases risk (43) and reduces survival (187) in pancreatic cancer; however the mechanisms are not fully understood. These results support our hypothesis, we found that obesity increases MDSCs in pancreatic tumor bearing mice and likely contributes to the immunosuppressive environment, which can enhance tumor growth. We found that obesity significantly enhanced tumor growth and reduced survival in Panc.02 tumor-bearing mice. Obesity also reduced the percentage of B cells, $CD4^+$ T cells, $CD8^+$T cells and increased macrophages in comparison to lean mice. Likewise, we found that obese mice had a greater expansion of MDSCs in the spleen, tumor and adipose in

comparison to lean mice and a greater expansion of MDSCs in the TDLN in comparison to lean and control mice. These findings are the first to demonstrate an obesity-induced expansion of MDSCs in lymphoid organs in a murine model of pancreatic cancer. In addition to the spleen, TDLN, and tumor, we also looked in the adipose tissue for MDSC accumulation. We found that obese mice accumulated more MDSCs in comparison to lean and control mice. We also found that adipose tissue from obese mice had increased gene expression of inflammatory mediators IL-6 and IL-10 which suggests the adipose tissue in obese mice may favor MDSC accumulation and tumor progression. This is the first study to demonstrate that immunosuppressive MDSCs accumulate in the adipose of obese Panc.02 tumor bearing mice.

The adipose tissue is known to be the site of obesity-induced inflammation via macrophage and adipose tissue production of pro-inflammatory mediators (e.g. IL-1β, TNF-α, IL-6) adipokines (e.g adipoenctin, leptin), and chemokines (MIP-1α and MCP-1) and other inflammatory mediators (NO and PGE_2) (57). MDSCs are also known to polarize macrophages toward a tumor-promoting phenotype via the production of IL-10 (16). These results suggest MDSCs are important in obesity-induced inflammation and tumor growth. We further characterized MDSCs subsets in lean, control, and obese mice. We found that obesity-induced a greater expansion of both MDSCs subsets in the spleen and a greater expansion of the monocytic subset in the tumor; however we observed that MDSC subset specific expansion varies by organ.

We also wanted to determine if MDSC subsets from lean, control and obese mice differed in their capacity to inhibit T cell function. MDSC subsets from lean, control, and obese mice suppressed T cell proliferation to the same degree. Obesity is known to induce a state of chronic inflammation, and although not statistically significant, we did observe a trend towards increased plasma levels of IL-6, and TNF-α in obese animals in comparison to lean and control mice. Overall these results show that obesity is altering immune cell distribution in lymphoid organs, and that the obesity-induced increase in MDSCs contributes to enhanced pancreatic tumor growth. Though not statistically significant, elevated levels of IL-6 and IL-1β in obese mice may be responsible for the greater accumulation of MDSCs observed in obese animals. The lack of statistical significance could be due to confounding by the tumor, masking the true response of the aforementioned cytokines and growth factors. This data suggest that obesity is not altering the suppressive capacity of MDSCs. However, due to the multiple mechanisms of suppression utilized by MDSCs to inhibit immunity, having more MDSCs in the context of obesity and cancer may be associated with poor outcomes.

Future studies should focus on examining if depletion of MDSCs in obese mice reduces tumor growth, and restores or enhances survival, and reduces adipose driven inflammation. Utilizing the diet induced obesity model and the KRAS/IN4A transgenic murine models of PDAC; which mimics human disease (with the stepwise progression from intraepithelial neoplasia to PDAC) we can determine the contribution of MDSCs tumor growth, survival, and adipose driven

inflammation. We could determine if Gr-1 antibody depletion of MDSCs reduces tumor volume and extends survival in comparison to control animals. Also we could measure cytokines (IL-6, IL-10, IL-8, and TNF-α) in adipose tissue via gene expression to see if depletion of MDSCs alters adipose driven inflammation.

Additional clinical studies are also needed to determine the effect of obesity on MDSC accumulation in obese cancer patients in comparison to healthy controls. Future studies can isolate PMBCs from obese pancreatic cancer patients and healthy controls and characterize MDSC accumulation via flow cytometry. We can also measure serum levels of inflammatory mediators, to determine if pro-inflammatory mediators correlate with MDSC accumulation. Lastly, MDSCs subsets can be isolated from obese pancreatic cancer patients and healthy controls and the suppressive capacity of MDSCs can be determined. The suppressive capacity of MDSCs can also be determined by measuring gene expression of (ARG-1, iNOS, Cox-2, and IL-6,) and by measuring TGF-β and IL-10 from MDSCs *in vitro* and by measuring the ability of MDSCs from obese patients and healthy controls to inhibit T cell function be determined via an MLR.

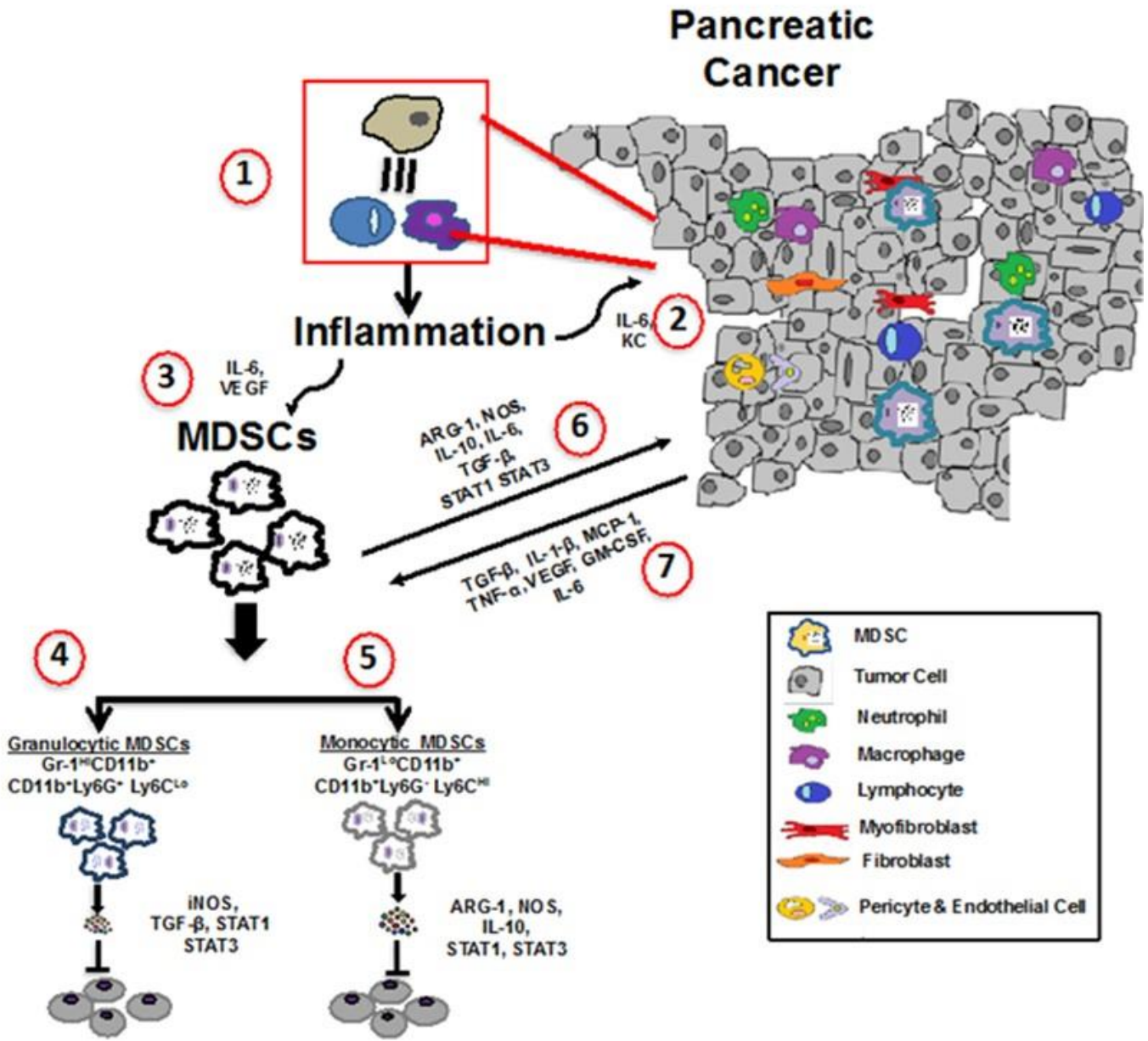
Pancreatic Cancer
1
Inflammation
IL-6, KC
2
3
IL-6, VEGF
MDSCs
ARG-1, NOS, IL-10, IL-6, TGF-β, STAT1 STAT3
6
TGF-β, IL-1-β, MCP-1, TNF-α, VEGF, GM-CSF, IL-6
7
4
5
Granulocytic MDSCs
Gr-1HiCD11b+
CD11b+Ly6G+ Ly6CLo
Monocytic MDSCs
Gr-1Lo CD11b+
CD11b+Ly6G- Ly6CHi
iNOS, TGF-β, STAT1 STAT3
ARG-1, NOS, IL-10, STAT1, STAT3
MDSC
Tumor Cell
Neutrophil
Macrophage
Lymphocyte
Myofibroblast
Fibroblast
Pericyte & Endothelial Cell

Figure 5.1. Mechanism of MDSC accumulation and function in the Panc.02 tumor model.
Pancreatic carcinogenesis is driven by tumor-induced immune suppression. (1) The interactions between tumor cells, immune cells and stromal cell can lead to a pro-inflammatory state via IL-6 and KC. (2) IL-6 and KC can further enhance pancreatic growth and progression. (3) Similarity, IL-6 and VEGF can also drive the expansion of MDSCs. A tumor-induced accumulation of MDSCs was evident and comparable using either the Gr-1/CD11b or the CD11b/Ly6C/Ly6G nomenclature. (4) The granulocytic Gr-1^{Hi}CD11b^{+} (CD11b^{+}Ly6G^{+}Ly6C^{Lo}) and monocytic Gr-1^{Lo}CD11b^{+} (CD11b^{+} Ly6C^{Hi}Ly6G^{-}) MDSCs both suppressed the function of T cells. Granulocytic Gr-1^{Hi}CD11b^{+} (CD11b^{+}Ly6G^{+}Ly6C^{Lo}) MDSCs expressed immunosuppressive factors iNOS, TGF-β, STAT1, and STAT3. (5) Monocytic Gr-1^{Lo}CD11b^{+} (CD11b^{+} Ly6C^{Hi}Ly6G^{-}) MDSCs expresses iNOS, ARG-1, IL-10, STAT1, and STAT3. (6) These immunosuppressive factors can potentiate pancreatic tumor growth. (7) Pancreatic cancer increases levels GM-CSF and VEGF, and inflammatory mediators IL-6, TNF-α, MCP-1, MIP-1α, and TGF-β, which can also enhance pancreatic tumor growth.

Results from study 1 demonstrate that pancreatic cancer can drive the accumulation of MDSCs (Figure 5.1). IL-6 and IL-8 drive pancreatic carcinogenesis. IL-6 and VEGF promote the accumulation of MDSCs from the bone marrow. MDSCs travel from bone marrow lymphoid organs, as a result of inflammatory signals. Splenic MDSC accumulation is a direct result of the elevated cytokines (TGF-β, IL-1β, IL-6, and TNF-α) chemokines (MCP-1, and MIP-1α) and growth factors (GM-CSF and VEGF) in the plasma of Panc.02 tumor bearing mice. Inflammatory mediators can be from immune cells, adipose tissue, tumor cells and stromal cells. Both MDSC subsets expand in this tumor model, with a preferential expansion of the granulocytic MDSC subset in comparison to the monocytic MDSC subset. The granulocytic and monocytic subsets inhibited the proliferation of T cells possibly though the production of IL-

10 and TGF-β. Granulocytic MDSCs express iNOS, TGF-β, STAT1, and STAT3. Monocytic MDSCs express ARG-1, iNOS, IL-10, STAT1, and STAT3. The production of the aforementioned factors from MDSCs can exacerbate pancreatic tumor growth.

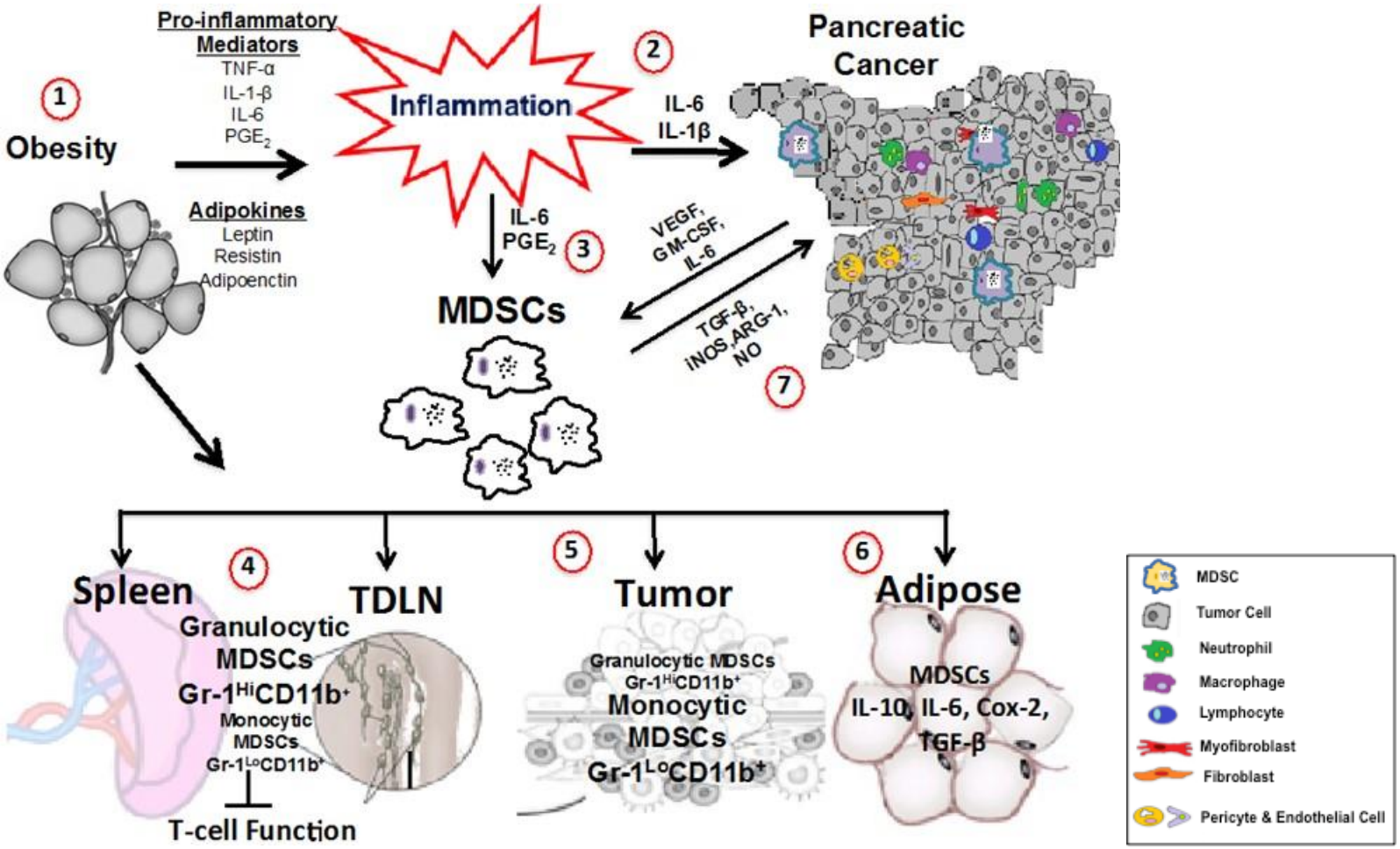
Pro-inflammatory Mediators
TNF-α
IL-1-β
IL-6
PGE2
1
Obesity
Adipokines
Leptin
Resistin
Adipoenctin
Inflammation
2
IL-6
IL-1β
Pancreatic Cancer
IL-6
PGE2
3
MDSCs
VEGF, GM-CSF, IL-6
TGF-β, INOS,ARG-1, NO
7
4
Spleen
TDLN
Granulocytic MDSCs
Gr-1HiCD11b+
Monocytic MDSCs
Gr-1LoCD11b+
T-cell Function
5
Tumor
Granulocytic MDSCs
Gr-1HiCD11b+
Monocytic MDSCs
Gr-1LoCD11b+
6
Adipose
MDSCs
IL-10, IL-6, Cox-2, TGF-β
MDSC
Tumor Cell
Neutrophil
Macrophage
Lymphocyte
Myofibroblast
Fibroblast
Pericyte & Endothelial Cell

Figure 5.2. Mechanisms underlying obesity induced expansion of MDSCs in the Panc.02 model.
(1) Infiltration of immune cell into the adipose leads to production of pro-inflammatory mediators and adipokines, which creates a state of low-grade chronic inflammation. (2) This low-grade chronic inflammation can drive pancreatic carcinogenesis via the production of pro-inflammatory mediators IL-6 and IL-1β. (3) Increased serum levels of VEGF, GM-CSF, and IL-6 in Panc.02 tumor bearing mice can also drive the expansion of MDSCs. Similarly IL-6 and PGE_2 from the low grade chronic inflammation observed in obesity may also drive the expansion of MDSCs. (4) Obesity-induced MDSC accumulation in the spleen, TDLN, tumor and adipose. In the spleen and the TDLN we observed a preferential expansion of the Granulocytic MDSCs ($Gr\text{-}1^{Hi}CD11b^{+}$). (5) In the tumor we noted a preferential expansion of the monocytic subset ($Gr\text{-}1^{Lo}CD11b^{+}$). MDSCs subsets from the spleen inhibited T cell proliferation. (6) MDSCS were expanded in the adipose tissue, accompanied by the production of immunosuppressive factors IL-10, IL-6, Cox-2, and TGF-β. (7) The production of these immunosuppressive factors from MDSCs can potentiate pancreatic cancer growth.

Results from study 2 demonstrate an obesity-induced expansion of MDSCs in lymphoid organs, adipose and tumors of Panc.02 tumor bearing mice (Figure 5.2). Obesity induced inflammation occurs via cross talk between macrophages and adipokines in adipose tissue. Obesity-associated inflammation leads to production of pro-inflammatory mediators TNF-α, IL-1β, IL-6, PGE_2 and production of adipokines leptin, resistin and adiponectin, which can leads to pancreatic cancer. Obesity induced inflammation and tumor induced inflammation can both drive the expansion of MDSCs from the bone marrow through production of IL-6 and PGE_2. Obesity induced MDSC accumulation in the spleen, TDLN, tumor and adipose tissue. A preferential expansion of granulocytic MDSCs in spleen and TDLN in comparison to monocytic MDSCs. In the tumor, a preferential expansion of monocytic MDSCs is shown in comparison to granulocytic MDSCs. MDSCs accumulated in adipose tissue of obese

animals, and increased the production of IL-10, IL-6, and Cox-2 in the adipose tissue. Creating a niche for tumor promotion and immune suppression. Obesity induced MDSCs subsets in spleen that inhibited T cell proliferation. Obesity-induced MDSCs contribute to tumor growth in pancreatic cancer.

Targeting MDSCs for immunotherapy may offer promising results in extending survival and enhancing antitumor responses in cancer patients. In preclinical models, a variety of strategies have been used to either deplete MDSCs, block the development of MDSCs, differentiate MDSCs, and deactivate MDSCs (209). Administration of all trans retinoic acid (ATRA), a metabolite of vitamin A was shown to decrease numbers of MDSCs in both animals and humans tumor models. ATRA also enhances $CD8^+$ T cell responses in several preclinical models of cancer. In murine models of colon cancer, and in renal cell carcinoma patients' administration of ATRA showed a therapeutic effect by causing the differentiation of MDSCs in mature myeloid cells. It was reported that the neutralization of ROS by glutathione, one of the major antioxidants in the body was the mechanisms underlying the aforementioned effect (210).

Other studies have used targeted depletion of MDSCs as a therapeutic approach. 5-Fluorouracil and gemcitabine, both chemotherapeutic agents selectively deplete MDSCs in lymphoid organs and tumors of mice with mammary cancer (146, 172). In addition to therapeutic approaches aimed to differentiate or delete MDSCs studies have also tried to inhibit signaling molecules associated with MDSC accumulation and function. Sildenafil a phosphodieterase type 5 inhibitor was shown to down regulate MDSCs via an IL-

4Rα mechanism in lung, prostate, and melanoma tumor models. Whereas sunitinib a tyrosine kinase inhibitor decreased MDSC accumulation and function in renal cell carcinoma patients (105). Sunitinib also decreases MDSC accumulation and improves cancer vaccine efficacy in melanoma model of cancer (211). Thus the elimination, deactivation, and differentiation of MDSCs immunotherapy seem to offer promising results in the aforementioned studies. Cancer therapy should include elimination tumor cells, and blunt the effects of immune suppressive cells i.e. MDSCs, in addition to cancer therapy should activate antigen specific anti-tumor immune mechanisms. Optimal therapy for cancer patients should be a combination of therapies including, chemotherapy, radiotherapy, and immunotherapy.

APPENDIX

Adiponectin reduces proliferation of Panc.02 cells and augments TGF-β cytokine production

Abstract

Obesity is associated with an increased risk and reduced survival from many cancer types, including pancreatic cancer. There are many potential biological pathways linking obesity to pancreatic cancer including changes in growth factors, metabolic mediators, inflammatory mediators, immune mediators and adipokines. However the mechanisms are not fully understood. Adiponectin (APN), an adipokine secreted from adipose tissue exerts anti-inflammatory properties. Therefore, the goal of the current study was to evaluate the effect of APN on pancreatic cancer cells. We utilized a pancreatic cancer cell line (Panc.02) to determine the direct effects of APN on tumor growth. Panc.02 cells were treated with full-length and globular APN at a high (10μg/mL) and low (1μg/mL) concentration to mimic concentrations observed in normal weight and obese individuals, respectively. C57/BL6 mice were injected with $1x10^6$ Panc.02 cells s.c. in the left lumbar region. Tumor growth and survival were monitored three times a week starting at day 13 post tumor implantation. Animals were followed until 60 days post tumor implantation for endpoint analysis or until animal reached ethical limit for tumor volume ($1.5cm^3$). *In vitro,* both full-length and globular APN (10μg/mL) significantly reduced proliferation of Panc.02 cells. Full-length APN significantly reduced TGF-β secretion from Panc.02 cells. Plasma levels of APN were significantly reduced and TGF-β levels were significantly increased in Panc.02 animals in comparison to tumor free (TF) mice. These data suggest that APN is augmenting function of Panc.02 cells, and exerting anti-tumorigenic effects.

Introduction

Obesity is a risk factor for many cancers, including pancreatic cancer (43, 44, 90, 194-197). Recent epidemiology studies show obesity is associated with a reduction in survival from pancreatic cancer (92, 101, 187). Similar studies have been performed in pre-clinical models of obesity and pancreatic cancer (96, 198) and obesity increases tumor growth and reduces survival in all models studied. There are several potential pathways linking obesity and pancreatic cancer including hypoxia, insulin resistance, reactive oxygen species, chronic inflammation, and adipokines (212).

Obesity is characterized by low-grade chronic inflammation with increased inflammatory mediators (e.g. IL-1α, IL-1β, IL-6, TNF-α) and decreased levels of APN (57). APN is secreted from adipose tissue and exists as a full-length protein (full-length/recombinant), and as a proteolytic fragment (globular form). Full length APN can exist as a low molecular weight trimer, a middle molecular weight hexamer, and a high molecular weight trimer (213). Plasma levels of APN are inversely correlated with body mass index/visceral obesity (57). Normal circulating levels of APN range from 3-30µg/mL (214) in the human and 3-15µg/mL in the mouse.

The relationship between APN and pancreatic cancer progression remains unclear. Studies in the literature yield conflicting results clinical. In some studies serum levels of APN increase (105-107) and (215-217) in other studies levels of APN decrease in pancreatic cancer patients. In preclinical studies, APN can inhibit apoptosis of human and murine pancreatic cancer cell lines through the

APN receptor 1. APN can exert its biological effects through two different receptors, APN receptor 1 and APN receptor 2. The globular form of APN binds with a higher affinity to APN receptor 1 and the full-length form of APN binds with a greater affinity to APN receptor 2 (218). Activation of APN receptors can activate downstream signaling of AMPK and PPAR respectively (57). Huang *et al.* show that pancreatic tumor growth is reduced in APN KO mice in comparison to WT (wild type) controls in a orthotopic model of pancreatic cancer (219). These results suggest that APN deficiency can improve tumor growth. Contrasting the previous results, Kato *et al*. show that APN KO mice have larger tumors in comparison to WT controls in an orthotopic model of pancreatic cancer (104). Kato *et al.* also show that full-length APN inhibits proliferation of pancreatic cancer cells, and increases apoptosis and caspase activity in *vitro* (104). Discrepancies in the aforementioned studies could be due that Huang *et al.* measured tumor volume at 2 weeks, whereas Kato *et al.* measure tumor volume at 6 weeks. Due to the conflicting results in clinical and pre-clinical studies, the role of APN on pancreatic tumor growth is unclear.

The goal of the current study was to determine the direct effects of APN on pancreatic cancer growth. A better understanding of the relationship between APN and pancreatic cancer is important in understanding the biological mechanisms underlying obesity-induced tumor growth and progression.

Materials and Methods

Cell Culture

The murine ductal adenocarcinoma cell line Panc.02 was obtained from Dr. Jeffrey Schlom, (National Cancer Institute, National Institute of Health) and cultured in McCoy's 5A without G418 (Thermo Scientific; Logan, UT), and supplemented with 5% FBS (Gemini Bio Products; Sacramento, CA), 0.1mM non-essential amino acids (Mediatech; Manassas, VA), 1mM sodium pyruvate (Mediatech), 2mM glutamine (Mediatech), 10mM HEPES (Mediatech), 100U/mL penicillin streptomycin (Mediatech). Panc.02 Cells were maintained at 37°C and 5% CO2 and passaged every 3-4 days using trypsin/EDTA (Mediatech). Full-length and globular (AVISCERA BIOSCIENCE; Santa Clara, CA) APN were pre-treated with proteinase K (100ug/mL) for 1 hour at 37°C prior to usage (Ambion; Applied Biosystems, Waltham, MA).

Flow cytometric analyses

Panc.02 cells were harvested using trypsin/EDTA and washed twice in PBS at 4°C. Cells were incubated with Fc block (Biolegend) and $1x10^6$ cells were stained with saturating concentrations of conjugated antibodies for 30 min at 4° C, as previously described (177). Following incubation with the conjugated antibodies, cells were washed twice in PBS and fixed in 1% paraformaldehyde for 15 mins at 4° C. Cells were washed twice with 1X wash perm buffer (BD). Cells were then incubated in 1X wash perm buffer (BD) for 15 mins and antibodies to adiponectin receptors 1 (Biorbyt; Cambridgeshire, United Kingdom) and 2 (Biorbyt) were added for 30 min at 4° C. Cells were washed with 1X wash perm buffer (BD). Cells were gated on forward vs. side scatter for a total of 20,000 events. All flow cytometric analyses were performed on a Beckman Coulter FC500 flow

cytometer (Beckman Coulter; Indianapolis, IN). Flow cytometric analyses were plotted and analyzed using Flow Jo software (Tree Star; Ashland, OR).

Cytokine Measurements

Panc.02 cells ($1x10^6$) were treated with vehicle control (VC), or both forms of APN (0, 1ug/mL, 10ug/mL) for 24 hrs. TGF-β (Biolegend) and APN (Abcam) was assessed cell culture supernatants via ELISA according to manufactures instructions and quantified on an Epoch Microplate Spectrophotometer (Biotek; Winooski, VT). Each assay was performed in duplicate.

Cell Proliferation

Panc.02 cells ($1x10^5$) were treated with VC or both forms of APN (0,1ug/mL, 10ug/mL) and incubated in flat-bottomed, 96-well plates and were pulsed with 1µCi per well of tritiated thymidine at 48 hours. Proliferation was assessed by tritiated thymidine (H3) incorporation at 72 hours (Perkin Elmer; Waltham, MA) on a microbeta plate reader (Perkin Elmer; Waltham, MA). Each assay was performed in triplicate.

Statistics

All data are presented as the mean plus or minus the standard error mean. Differences in APN receptor expression and plasma cytokine production were assessed between groups via a Student t-test or the Mann-Whitney test, depending on normality and variance. Differences in cytokine data from cell culture supernatants were determined using one-way ANOVA, followed by Dunn's or Bonferroni correction for multiple comparisons where appropriate. All analyses were conducted using GraphPad Prism 5 software (GraphPad

Software; La Jolla, CA) and statistical significance was accepted at the p≤0.05 level.

Results

APN reduces the proliferation of Panc.02 cells

Both full-length and globular APN significantly reduced the proliferation of Panc.02 cells (Figure 1a; 2-ANOVA, $F_{(2, 192)}$= 21.19, p≤0.001). Full-length APN (10µg/mL and 20µg/mL) significantly reduced proliferation of Panc.02 cells in comparison to VC (Bonferroni multiple comparison, p<0.001). Globular APN (10µg/mL) significantly reduced proliferation of Panc.02 cells in comparison to VC (Bonferroni multiple comparison, p<0.001). Globular APN (20µg/mL) significantly reduced proliferation of Panc.02 cells (Bonferroni multiple comparison, p<0.010) in comparison to VC.

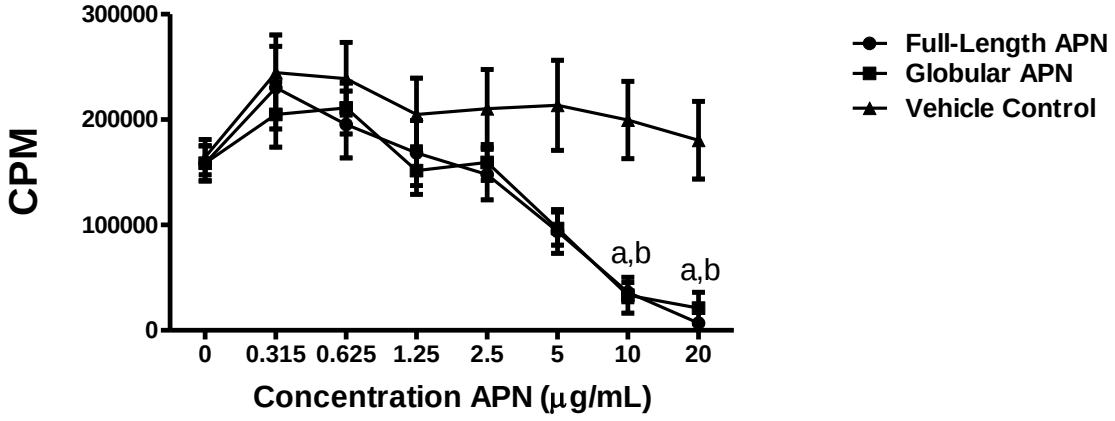

Fig. 1. APN reduces the proliferation of Panc.02 cells. Panc.02 cells were treated with VC, or both forms of APN (0, 1ug/mL, 10 µg/mL). Proliferation was assessed after 72 hours in culture using a thymidine incorporation assay. Both forms of APN (10 and 20µg/mL) significantly reduced proliferation of Panc.02 cells (Figure 1, 2-Way ANOVA

shows a significant interaction of treatment x concentration of APN $F_{(2,192)}$=21.19, p≤0.001). Results are from 3 independent experiments. a indicates a significant difference in full-length APN in comparison to. VC. b indicates a significant difference in globular APN in comparison to VC.

Panc.02 cells deferentially express APN receptors

Representative histogram overlay of APN receptor expression of Panc.02 cells (Figure 2a). Panc.02 cells expressed both APN receptors, however they expressed significantly more of APN receptor 1 (Figure 2b; unpaired t test, p=0.066). A similar trend was observed for the mean fluorescence intensity (MFI) of APN receptor expression on Panc.02 cells. MFI of APN receptor 1 was higher in Panc.02 cells in comparison to APN receptor 2 (Figure 2c; unpaired t test, p=0.031).

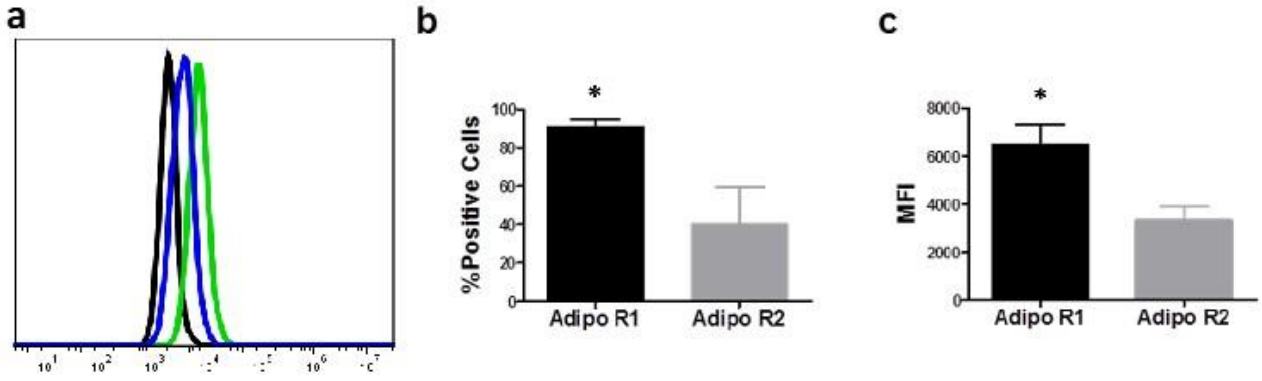

Fig. 2. Panc.02 cells deferentially express APN receptors. Panc.02 cells treated with VC, or both forms of APN (0, 1ug/mL, 10ug/mL) for 24 hrs. and analyzed via flow cytometry for expression of APN receptors. (A) Representative histogram of Panc.02 cells treated with VC, or both forms of APN (0, 1ug/mL, 10ug/mL) for 24 hrs. (b) Panc.02 cells express significantly more APN receptor and 1 in comparison to APN receptor 2 (Figure 2b; unpaired t test, p=0.066). (c) APN receptor 1 expressed a significantly higher MFI in comparison to APN receptor 2 (Figure 2c; unpaired t test, p=0.031). Results are

from 3 independent experiments. Asterisk indicates a significant difference between groups.

APN reduces TGF-β secretion from Panc. 02 cells

10μg/mL of full-length APN significantly reduced the secretion of TGF-β from Panc.02 cells in comparison to VC (Figure 3b; Kruskal Wallis, κ =11.15, p=0.011). Dunn's multiple comparison test: 0μg vs. 10μg full-length APN (p<0.050) and VC vs. 10μg full-length APN (p≤0.010).

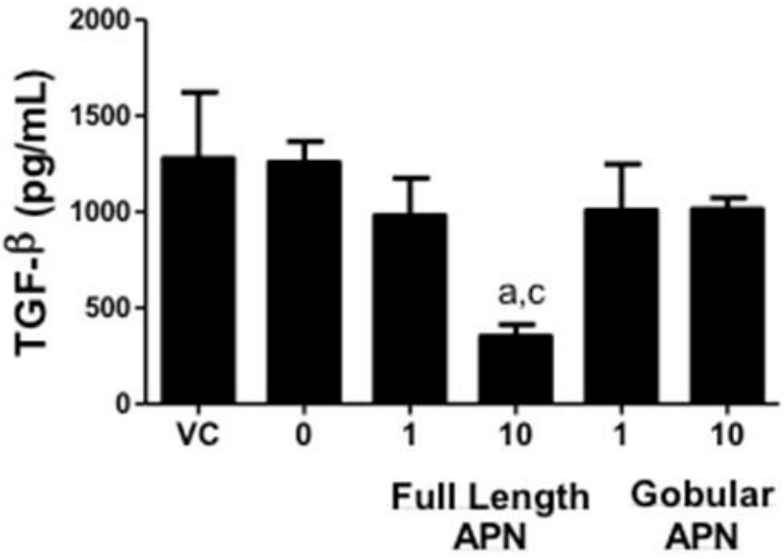

Fig. 3. APN augments TGF-β secretion from Panc.02 cells. Culture supernatants were harvested from Panc.02 cells treated with VC, or both forms of APN (0, 1μg/mL, 10μg/mL) for 24 hrs. ELISA was used to measure TGF-β production. 10μg/mL of full-length APN significantly reduced the secretion of TGF-β from Panc.02 cells (Figure 3b; Kruskal Wallis, κ =11.15, p=0.011). Dunn's multiple comparison test: 0μg vs. 10μg full-length APN (p<0.050) and VC vs. 10μg full-length APN (p≤0.010). a indicates a significant difference from 0ug/mL APN, c indicates a significant difference from vehicle control. Results are from 3 independent experiments. a indicates a significant difference from 0ug/mL APN, c indicates a significant difference from vehicle control. Results are from 3 independent experiments.

Plasma levels of APN and TGF-β levels are altered in Panc.02 tumor-bearing mice

Panc.02 tumor-bearing mice had significantly lower levels of APN (Figure. 4a, Mann Whitney test, p=0.045) and significantly higher levels of TGF-β (Figure. 4b, Mann Whitney test, p=0.012) in plasma in comparison TF animals.

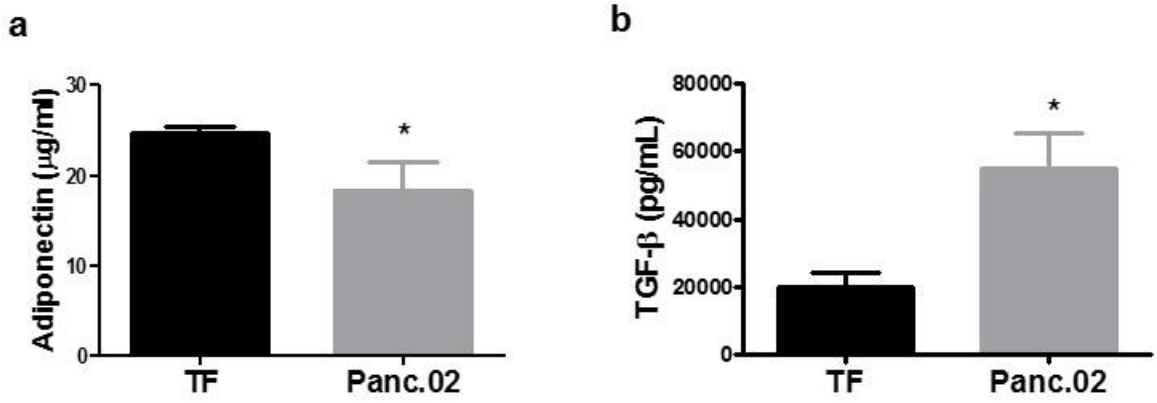

Fig. 4. Plasma levels of APN and TGF-β levels are augmented in Panc.02 tumor-bearing mice. Plasma levels of APN (Figure. 2a) and TGF- β (Figure.2b) were measured in TF (n=4/group) and Panc.02 tumor bearing (n=10/group) animals using an ELISA at day 60-post tumor implantation. Panc.02 tumor bearing animals had significantly higher levels of APN (Figure. 4a, Mann Whitney test, p=0.045) and significantly higher levels of TGF-β (Figure. 4b, Mann Whitney test, p=0.012) in plasma in comparison TF animals. Asterisk indicates a significant difference between groups.

Discussion

In this study we demonstrated that both the full-length and globular APN inhibited the proliferation of Panc.02 cells. We also showed Panc.02 cells have a preferential expression of APN receptor 1. Additionally full-length APN reduced TGF-β secretion from Panc.02 cells. Lastly we observed a reduction in plasma levels of APN and increased plasma level of TGF-β in Panc.02 tumor bearing

mice in comparison to TF controls. Thus, APN alters the proliferation and production of immunosuppressive factor TGF-β in pancreatic cancer cells, which may play a role in the association between obesity and pancreatic cancer.

Over 64% of the U.S. adult population is either overweight or obese (51). Obesity increases the risk pancreatic cancer (1) however the mechanisms underlying the link between obesity and pancreatic cancer are not fully understood. APN is one of the most abundant adipocytokine found in circulation (220). Plasma levels of APN are inversely correlated with body mass index/visceral obesity. Previous studies have established that APN has anti-atherogenic (102) insulin sensitizing (221) and immunomodulatory effects (221). The role of APN in pancreatic cancer growth is not fully understood.

Our interpretation of the results addressing the biological role of APN has become complicated due to the different activity of full length APN in comparison to globular APN. APN mediates its effects via two receptors, APN receptor 1 and APN receptor 2. Globular APN preferentially binds to the APN receptor 1 receptor resulting in AMPK (5’ AMP-activated protein kinase) activation, whereas full length APN binds to APN receptor 2 and is associated with activation of PPAR-α (peroxisome proliferator- activated receptor alpha) pathway(57). APN receptors are expressed in breast (222), colon (103) and pancreatic (219) cancer cell lines. In our study we also observed expression of both receptors, with a preferential expression of APN receptor 1 in Panc.02 cells, which is consistent with the literature (219).

APN has potent anti-inflammatory effects. Short-term treatment with APN on Raw 264.7 macrophages increases TNF-α and IL-10 production (100). APN also inhibited NF-kB activation in human aortic endothelial cells (102). NF-kB is an important pathway, regulating survival, proliferation, inflammation, and immune regulation. Full length APN promoted a shift in the phenotype of M1 and M2 macrophages towards a more anti-inflammatory phenotype (223). APN also induced the production of anti-inflammatory cytokines IL-10 and IL-1RA in human monocytes, macrophages, and DCs (224). Kusunoki and colleagues demonstrate that APN stimulates the production of prostaglandin E2 (PGE_2) in synovial fibroblasts (58). Lee and colleagues demonstrate a synergistic effect between APN and pro-inflammatory mediators IL-1β, IL-6, IL-8 and COX-2 in mesenchymal cells from patients with rheumatoid arthritis (RA) (225). These studies suggest a relationship between inflammatory mediators, and adipokines using a model of chronic inflammation. Collectively, these studies demonstrate APNs anti-inflammatory effects on macrophages, endothelial cells and mesenchymal cells. The later studies suggest that APN may be involved in the pathogenesis of RA (225).

In murine tumor models, APN has an anti- proliferative effect on colon cancer cell lines (101). Lang and colleagues demonstrate that APN promotes the migration of colon cancer cells. Globular APN inhibits the proliferation of colon cancer cell through activation of AMPK and reduction in the mTOR (mechanistic target of rapamycin) pathway (103). Globular APN also inhibited proliferation of breast cancer cell line via JNK2 (protein kinase) signaling (222). In murine

pancreatic cancer models, full-length APN inhibited proliferation on pancreatic cancer cells, and increased apoptosis and caspase activity in *vitro* (104). Similar to the aforementioned study, we observed both full-length and globular APN inhibited proliferation of Panc.02 cells. Our results are similar to Kato *et al.* however interpretations of results must be carefully considered, as the relationship between APN and cancer is very complex, and tumor model dependent.

The inflammatory mediator TGF-β is also implicated in pancreatic carcinogenesis. Plasma levels of TGF-β1 in pancreatic cancer patients are associated with advanced disease and reductions in survival (226). Pancreatic cancer patients also have increases in expression of TGF-β receptors 1 and 2 in comparison to normal controls (227). This is the first report to demonstrate that APN reduced TGF-β production from Panc.02 cells at levels, which would be observed in normal weight individuals. These studies suggest an anti-tumorigenic effect of APN on pancreatic cancer. The reduction in TGF-β production suggests that APN may offer protective effects in normal weight pancreatic cancer patients by reduction production of the immunosuppressive factors.

The relationship between APN and pancreatic cancer progression remains unclear. Studies in the literature yield conflicting results for plasma levels of APN in pancreatic cancer patients (105-107). We demonstrated that plasma levels of APN are significantly reduced in Panc.02 tumor bearing animals in comparison to TF controls. However additional clinical studies are needed to confirm if levels of APN are increased or decreased in pancreatic cancer patients.

APN has potent anti-inflammatory effects. We demonstrate here an anti-tumorigenic effect of APN on Panc.02 cells. APN reduced proliferation and TGF-β cytokine production in/from Panc.02 cells. These results suggest that the absence/reduction of APNs anti-inflammatory effect observed in obese individuals may be responsible for some effects of chronic inflammation as seen with obesity-related cancers. These results shed light into the complex interface between obesity, and pancreatic cancer. Future studies are warranted to understand the complex relationship between APN and pancreatic carcinogenesis.

References

1. Aune, D., et al., *Body mass index, abdominal fatness and pancreatic cancer risk: a systematic review and non-linear dose-response meta-analysis of prospective studies.* Ann Oncol, 2012. **23**(4): p. 843-52.
2. Arslan, A.A., et al., *Anthropometric measures, body mass index, and pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan).* Arch Intern Med, 2010. **170**(9): p. 791-802.
3. Jiao, L., et al., *Body mass index, effect modifiers, and risk of pancreatic cancer: a pooled study of seven prospective cohorts.* Cancer Causes Control, 2010. **21**(8): p. 1305-14.
4. Genkinger, J.M., et al., *A pooled analysis of 14 cohort studies of anthropometric factors and pancreatic cancer risk.* Int J Cancer, 2011. **129**(7): p. 1708-17.
5. Renehan, A.G., et al., *Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies.* Lancet, 2008. **371**(9612): p. 569-78.
6. Larsson, S.C., N. Orsini, and A. Wolk, *Body mass index and pancreatic cancer risk: A meta-analysis of prospective studies.* Int J Cancer, 2007. **120**(9): p. 1993-8.
7. Berrington de Gonzalez, A., S. Sweetland, and E. Spencer, *A meta-analysis of obesity and the risk of pancreatic cancer.* Br J Cancer, 2003. **89**(3): p. 519-23.
8. McWilliams, R.R., et al., *Obesity adversely affects survival in pancreatic cancer patients.* Cancer, 2010. **116**(21): p. 5054-62.
9. Genkinger, J.M., et al., *Central adiposity, obesity during early adulthood, and pancreatic cancer mortality in a pooled analysis of cohort studies. LID - mdv355 [pii].* (1569-8041 (Electronic)).
10. Kasenda B Fau - Bass, A., et al., *Survival in overweight patients with advanced pancreatic carcinoma: a multicentre cohort study.* (1471-2407 (Electronic)).
11. Lashinger, L.M., et al., *Dietary energy balance modulation of Kras- and Ink4a/Arf+/--driven pancreatic cancer: the role of insulin-like growth factor-I.* (1940-6215 (Electronic)).
12. Bao, B., et al., *The complexities of obesity and diabetes with the development and progression of pancreatic cancer.* Biochim Biophys Acta, 2011. **1815**(2): p. 135-46.
13. Tilg, H. and A.R. Moschen, *Adipocytokines: mediators linking adipose tissue, inflammation and immunity.* Nat Rev Immunol, 2006. **6**(10): p. 772-83.
14. Kelesidis, I., T. Kelesidis, and C.S. Mantzoros, *Adiponectin and cancer: a systematic review.* Br J Cancer, 2006. **94**(9): p. 1221-5.
15. Simpson, F. and J.P. Whitehead, *Adiponectin--it's all about the modifications.* Int J Biochem Cell Biol, 2010. **42**(6): p. 785-8.

16. Dalamaga, M., et al., *Pancreatic cancer expresses adiponectin receptors and is associated with hypoleptinemia and hyperadiponectinemia: a case-control study.* (1573-7225 (Electronic)).
17. Chang, M.C., et al., *Adiponectin as a potential differential marker to distinguish pancreatic cancer and chronic pancreatitis.* (1536-4828 (Electronic)).
18. Krechler T Fau - Zeman, M., et al., *Leptin and adiponectin in pancreatic cancer: connection with diabetes mellitus.* (0028-2685 (Print)).
19. Kato, M., et al., *Adiponectin inhibits murine pancreatic cancer growth.* (1573-2568 (Electronic)).
20. Turbitt, W.J., et al., *Fish Oil Enhances T Cell Function and Tumor Infiltration and Is Correlated With a Cancer Prevention Effect in HER-2/neu But Not PyMT Transgenic Mice.* Nutr Cancer, 2015. **67**(6): p. 965-75.
21. Flegal, K.M., et al., *Prevalence and trends in obesity among US adults, 1999-2000.* (0098-7484 (Print)).
22. Grossmann, M.E., et al., *Effects of adiponectin on breast cancer cell growth and signaling.* (0007-0920 (Print)).
23. Fujisawa, T., et al., *Adiponectin suppresses colorectal carcinogenesis under the high-fat diet condition.* Gut, 2008. **57**(11): p. 1531-8.
24. Huang, H., et al., *Mechanisms for the anti-inflammatory effects of adiponectin in macrophages.* J Gastroenterol Hepatol, 2008. **23 Suppl 1**: p. S50-3.
25. Ouchi, N., et al., *Adiponectin, an Adipocyte-Derived Plasma Protein, Inhibits Endothelial NF- B Signaling Through a cAMP-Dependent Pathway.* Circulation, 2000. **102**(11): p. 1296-1301.
26. Lovren, F., et al., *Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages.* Am J Physiol Heart Circ Physiol, 2010. **299**(3): p. H656-63.
27. Wolf, A.M., et al., *Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes.* Biochem Biophys Res Commun, 2004. **323**(2): p. 630-5.
28. Javle, M., et al., *Biomarkers of TGF-beta signaling pathway and prognosis of pancreatic cancer.* (1932-6203 (Electronic)).
29. Lu, Z., et al., *Presence of two signaling TGF-beta receptors in human pancreatic cancer correlates with advanced tumor stage.* (0163-2116 (Print)).

References

1. Society, A.C., *Cancer facts & figures.* The Society, 2014. Atlanta, GA.
2. Wormann, S.M., et al., *The immune network in pancreatic cancer development and progression.* (1476-5594 (Electronic)).
3. Bo, Y., *Immunotherapy of the Pancreatic Cancer, Pancreatic Cancer - Clinical Management*. 2012.
4. Teague, A., K.H. Lim, and A. Wang-Gillam, *Advanced pancreatic adenocarcinoma: a review of current treatment strategies and developing therapies.* (1758-8340 (Print)).
5. Swann, J.B. and M.J. Smyth, *Immune surveillance of tumors.* (0021-9738 (Print)).
6. Dunn, G.P., R.D. Old Lj Fau - Schreiber, and R.D. Schreiber, *The three Es of cancer immunoediting.* (0732-0582 (Print)).
7. Yu, X., Ji, Shunrong, in Xu, Wantong Yao, Bin Qu, Wenwei Zhu, Wenyan Xu, Bo Zhang and Yongfeng Xu, *CD8+ T Cells Are Compromised In Human Pancreatic Cancer.* Translation Medicine, 2012.
8. Fukunaga, A., et al., *CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma.* (1536-4828 (Electronic)).
9. Whiteside, T.L., *The tumor microenvironment and its role in promoting tumor growth.* (1476-5594 (Electronic)).
10. Roncarolo, M.G., et al., *Interleukin-10-secreting type 1 regulatory T cells in rodents and humans.* (0105-2896 (Print)).
11. Serafini, P., et al., *High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells.* (0008-5472 (Print)).
12. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system.* Nat Rev Immunol, 2009. **9**(3): p. 162-74.
13. Kusmartsev, S. and D.I. Gabrilovich, *Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species.* (0741-5400 (Print)).
14. Tadmor, T., D. Attias, and A. Polliack, *Myeloid-derived suppressor cells--their role in haemato-oncological malignancies and other cancers and possible implications for therapy.* Br J Haematol, 2011. **153**(5): p. 557-67.
15. Ostrand-Rosenberg, S. and P. Sinha, *Myeloid-derived suppressor cells: linking inflammation and cancer.* J Immunol, 2009. **182**(8): p. 4499-506.
16. Bunt, S.K., et al., *Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression.* Cancer Res, 2007. **67**(20): p. 10019-26.
17. Nagaraj, S., et al., *Mechanism of T cell tolerance induced by myeloid-derived suppressor cells.* J Immunol, 2010. **184**(6): p. 3106-16.
18. Katoh, H. and M. Watanabe, *Myeloid-Derived Suppressor Cells and Therapeutic Strategies in Cancer.* (1466-1861 (Electronic)).

19. Bunt, S.K., et al., *Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression.* J Immunol, 2006. **176**(1): p. 284-90.
20. Song, X., et al., *CD11b+/Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting cells.* J Immunol, 2005. **175**(12): p. 8200-8.
21. Sinha, P., et al., *Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response.* J Immunol, 2007. **179**(2): p. 977-83.
22. Sinha, P., et al., *Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells.* (0008-5472 (Print)).
23. Gabrilovich, D., et al., *Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo.* (0006-4971 (Print)).
24. Diaz-Montero, C.M., et al., *Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy.* (1432-0851 (Electronic)).
25. Gabitass, R.F., et al., *Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13.* (1432-0851 (Electronic)).
26. Bracci, P.M., *Obesity and pancreatic cancer: overview of epidemiologic evidence and biologic mechanisms.* Mol Carcinog, 2012. **51**(1): p. 53-63.
27. Markowitz, J., et al., *Patients with pancreatic adenocarcinoma exhibit elevated levels of myeloid-derived suppressor cells upon progression of disease.* (1432-0851 (Electronic)).
28. Basso, D., et al., *Pancreatic tumors and immature immunosuppressive myeloid cells in blood and spleen: role of inhibitory co-stimulatory molecules PDL1 and CTLA4. An in vivo and in vitro study.* (1932-6203 (Electronic)).
29. Bellone, G., et al., *Tumor-associated transforming growth factor-beta and interleukin-10 contribute to a systemic Th2 immune phenotype in pancreatic carcinoma patients.* (0002-9440 (Print)).
30. Stromnes, I.M., J.S. Brockenbrough, and K. Izeradjene, *Targeted depletion of an MDSC subset unmasks pancreatic ductal adenocarcinoma to adaptive immunity.* 2014. **63**(11): p. 1769-81.
31. Bayne, L.J., et al., *Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer.* Cancer Cell, 2012. **21**(6): p. 822-35.
32. Hasnis, E., et al., *Anti-Bv8 antibody and metronomic gemcitabine improve pancreatic adenocarcinoma treatment outcome following weekly gemcitabine therapy.* Neoplasia, 2014. **16**(6): p. 501-10.
33. Bunt, S.K., et al., *Rosiglitazone and Gemcitabine in combination reduces immune suppression and modulates T cell populations in pancreatic cancer.* (1432-0851 (Electronic)).

34. Nelson, N., et al., *Preparation of myeloid derived suppressor cells (MDSC) from naive and pancreatic tumor-bearing mice using flow cytometry and automated magnetic activated cell sorting (AutoMACS).* (1940-087X (Electronic)).
35. Peng, M., et al., *Embelin inhibits pancreatic cancer progression by directly inducing cancer cell apoptosis and indirectly restricting IL-6 associated inflammatory and immune suppressive cells.* (1872-7980 (Electronic)).
36. Husain, Z., et al., *Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells.* (1550-6606 (Electronic)).
37. Pilon-Thomas, S., et al., *Murine pancreatic adenocarcinoma dampens SHIP-1 expression and alters MDSC homeostasis and function.* (1932-6203 (Electronic)).
38. Porembka, M.R., et al., *Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth.* Cancer Immunol Immunother, 2012. **61**(9): p. 1373-85.
39. Kidiyoor, A., et al., *Pancreatic Cancer Cells Isolated from Muc1-Null Tumors Favor the Generation of a Mature Less Suppressive MDSC Population.* (1664-3224 (Electronic)).
40. Panni, R.Z., et al., *Tumor-induced STAT3 activation in monocytic myeloid-derived suppressor cells enhances stemness and mesenchymal properties in human pancreatic cancer.* (1432-0851 (Electronic)).
41. Karakhanova, S., et al., *Characterization of myeloid leukocytes and soluble mediators in pancreatic cancer: importance of myeloid-derived suppressor cells.* (2162-4011 (Print)).
42. *<Flegal, 2002.pdf>.*
43. Aune, D., et al., *Body mass index, abdominal fatness and pancreatic cancer risk: a systematic review and non-linear dose-response meta-analysis of prospective studies.* Ann Oncol, 2012. **23**(4): p. 843-52.
44. Genkinger, J.M., et al., *A pooled analysis of 14 cohort studies of anthropometric factors and pancreatic cancer risk.* Int J Cancer, 2011. **129**(7): p. 1708-17.
45. Greenberg, A.S. and M.S. Obin, *Obesity and the role of adipose tissue in inflammation and metabolism.* (0002-9165 (Print)).
46. Karlsson, E.A. and M.A. Beck, *The burden of obesity on infectious disease.* Exp Biol Med (Maywood), 2010. **235**(12): p. 1412-24.
47. Sheridan, P.A., et al., *Obesity is associated with impaired immune response to influenza vaccination in humans.* Int J Obes (Lond), 2012. **36**(8): p. 1072-7.
48. Bories, G., et al., *Impaired alternative macrophage differentiation of peripheral blood mononuclear cells from obese subjects.* (1752-8984 (Electronic)).
49. Laue, T., et al., *Altered NK cell function in obese healthy humans.* BMC Obes, 2015. **2**: p. 1.
50. Costanzo, A.E., et al., *Obesity impairs gammadelta T cell homeostasis and antiviral function in humans.* (1932-6203 (Electronic)).

51. Flegal, K.M., et al., *Prevalence and trends in obesity among US adults, 1999-2000.* (0098-7484 (Print)).
52. CDC, *Obesity Statistics.* 2014.
53. Dinarello, C.A., *Interleukin-1 in the pathogenesis and treatment of inflammatory diseases.* (1528-0020 (Electronic)).
54. Lalani, I., A.R. Bhol K Fau - Ahmed, and A.R. Ahmed, *Interleukin-10: biology, role in inflammation and autoimmunity.* (1081-1206 (Print)).
55. Warren, J.S., *Interleukins and tumor necrosis factor in inflammation.* (1040-8363 (Print)).
56. Kenneth, M., *Janeway's Immunobiology*. 2011.
57. Tilg, H. and A.R. Moschen, *Adipocytokines: mediators linking adipose tissue, inflammation and immunity.* Nat Rev Immunol, 2006. **6**(10): p. 772-83.
58. Kusunoki, N., et al., *Adiponectin stimulates prostaglandin E(2) production in rheumatoid arthritis synovial fibroblasts.* (1529-0131 (Electronic)).
59. Xu, H., et al., *Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance.* J Clin Invest, 2003. **112**(12): p. 1821-30.
60. Sullivan, J., et al., *Serum monocyte chemoattractant protein-1 in pancreatic cancer.* (1687-8469 (Electronic)).
61. Edwards, B.K., et al., *Annual report to the nation on the status of cancer, 1975-2002, featuring population-based trends in cancer treatment.* J Natl Cancer Inst, 2005. **97**(19): p. 1407-27.
62. Wroblewski, L.E., K.T. Peek Rm Jr Fau - Wilson, and K.T. Wilson, *Helicobacter pylori and gastric cancer: factors that modulate disease risk.* (1098-6618 (Electronic)).
63. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
64. Zyromski, N. *Obesity-Related Effects on Pancreatic Disease*. Pancreapedia: Exocrine Knowledge Base, 2015. DOI: 10.3998/panc.2015.20.
65. Lu, H., C. Ouyang W Fau - Huang, and C. Huang, *Inflammation, a key event in cancer development.* (1541-7786 (Print)).
66. Greten, F.R., et al., *IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer.* (0092-8674 (Print)).
67. Von Hoff Daniel B., E.B.D., Hruban Ralph, *Pancreatic Cancer*.
68. Braeuer, S.J., et al., *Constitutively activated nuclear factor-kappaB, but not induced NF-kappaB, leads to TRAIL resistance by up-regulation of X-linked inhibitor of apoptosis protein in human cancer cells.* (1541-7786 (Print)).
69. Garcia-Rodriguez, L.A. and C. Huerta-Alvarez, *Reduced risk of colorectal cancer among long-term users of aspirin and nonaspirin nonsteroidal antiinflammatory drugs.* (1044-3983 (Print)).
70. Gierach, G.L., et al., *Nonsteroidal anti-inflammatory drugs and breast cancer risk in the National Institutes of Health-AARP Diet and Health Study.* (1465-542X (Electronic)).

71. Warner, T.D., et al., *Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis.* (0027-8424 (Print)).
72. Elder, D.J., et al., *Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression.* (1078-0432 (Print)).
73. de Heredia, F.P., A. Gomez-Martinez S Fau - Marcos, and A. Marcos, *Obesity, inflammation and the immune system.* (1475-2719 (Electronic)).
74. Mertz, D., et al., *Populations at risk for severe or complicated influenza illness: systematic review and meta-analysis.* (1756-1833 (Electronic)).
75. Smith, A.G., et al., *Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus.* J Nutr, 2007. **137**(5): p. 1236-43.
76. Lautenbach, A., et al., *Altered phenotype of NK cells from obese rats can be normalized by transfer into lean animals.* (1930-7381 (Print)).
77. Winer, D.A., et al., *B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies.* (1546-170X (Electronic)).
78. DeFuria, J., et al., *B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile.* (1091-6490 (Electronic)).
79. Nishimura, S., et al., *CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity.* (1546-170X (Electronic)).
80. Chen, Y., et al., *Adipose tissue dendritic cells enhances inflammation by prompting the generation of Th17 cells.* (1932-6203 (Electronic)).
81. Smith, A.G., et al., *Selective impairment in dendritic cell function and altered antigen-specific CD8+ T-cell responses in diet-induced obese mice infected with influenza virus.* Immunology, 2009. **126**(2): p. 268-79.
82. Lumeng, C.N., et al., *Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity.* Diabetes, 2007. **56**(1): p. 16-23.
83. Xia, S., et al., *Gr-1+ CD11b+ myeloid-derived suppressor cells suppress inflammation and promote insulin sensitivity in obesity.* J Biol Chem, 2011. **286**(26): p. 23591-9.
84. Wagner, N.M., et al., *Circulating regulatory T cells are reduced in obesity and may identify subjects at increased metabolic and cardiovascular risk.* (1930-739X (Electronic)).
85. Deiuliis, J., et al., *Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers.* (1932-6203 (Electronic)).
86. Zeyda, M., et al., *Inflammation correlates with markers of T-cell subsets including regulatory T cells in adipose tissue from obese patients.* (1930-739X (Electronic)).

87. Hale, M., et al., *Obesity triggers enhanced MDSC accumulation in murine renal tumors via elevated local production of CCL2.* (1932-6203 (Electronic)).
88. Calle, E.E., et al., *The American Cancer Society Cancer Prevention Study II Nutrition Cohort: rationale, study design, and baseline characteristics.* Cancer, 2002. **94**(9): p. 2490-501.
89. Erarslan, E., et al., *Association of visceral fat accumulation and adiponectin levels with colorectal neoplasia.* Dig Dis Sci, 2009. **54**(4): p. 862-8.
90. Jiao, L., et al., *Body mass index, effect modifiers, and risk of pancreatic cancer: a pooled study of seven prospective cohorts.* Cancer Causes Control, 2010. **21**(8): p. 1305-14.
91. Renehan, A.G., et al., *Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies.* The Lancet, 2008. **371**(9612): p. 569-578.
92. Kasenda B Fau - Bass, A., et al., *Survival in overweight patients with advanced pancreatic carcinoma: a multicentre cohort study.* (1471-2407 (Electronic)).
93. Bethea, T.N., et al., *A pooled analysis of body mass index and pancreatic cancer mortality in african americans.* (1538-7755 (Electronic)).
94. Momi, N., et al., *Discovering the route from inflammation to pancreatic cancer.* (1121-421X (Print)).
95. Roberts, D.L., A.G. Dive C Fau - Renehan, and A.G. Renehan, *Biological mechanisms linking obesity and cancer risk: new perspectives.* (1545-326X (Electronic)).
96. Zyromski, N.J., et al., *Obesity potentiates the growth and dissemination of pancreatic cancer.* Surgery, 2009. **146**(2): p. 258-63.
97. Mendonsa, A.M., et al., *Modulation of the leptin receptor mediates tumor growth and migration of pancreatic cancer cells.* (1932-6203 (Electronic)).
98. Chin, C.C., et al., *Role of body mass index in colon cancer patients in Taiwan.* World J Gastroenterol, 2012. **18**(31): p. 4191-8.
99. Birmingham, J.M., et al., *Novel mechanism for obesity-induced colon cancer progression.* Carcinogenesis, 2009. **30**(4): p. 690-7.
100. Huang, H., et al., *Mechanisms for the anti-inflammatory effects of adiponectin in macrophages.* J Gastroenterol Hepatol, 2008. **23 Suppl 1**: p. S50-3.
101. McWilliams, R.R., et al., *Obesity adversely affects survival in pancreatic cancer patients.* Cancer, 2010. **116**(21): p. 5054-62.
102. Ouchi, N., et al., *Adiponectin, an Adipocyte-Derived Plasma Protein, Inhibits Endothelial NF- B Signaling Through a cAMP-Dependent Pathway.* Circulation, 2000. **102**(11): p. 1296-1301.
103. Fujisawa, T., et al., *Adiponectin suppresses colorectal carcinogenesis under the high-fat diet condition.* Gut, 2008. **57**(11): p. 1531-8.
104. Kato, M., et al., *Adiponectin inhibits murine pancreatic cancer growth.* (1573-2568 (Electronic)).

105. Dalamaga, M., et al., *Pancreatic cancer expresses adiponectin receptors and is associated with hypoleptinemia and hyperadiponectinemia: a case-control study.* (1573-7225 (Electronic)).
106. Chang, M.C., et al., *Adiponectin as a potential differential marker to distinguish pancreatic cancer and chronic pancreatitis.* (1536-4828 (Electronic)).
107. Krechler T Fau - Zeman, M., et al., *Leptin and adiponectin in pancreatic cancer: connection with diabetes mellitus.* (0028-2685 (Print)).
108. White, P.B., et al., *Insulin, leptin, and tumoral adipocytes promote murine pancreatic cancer growth.* J Gastrointest Surg, 2010. **14**(12): p. 1888-93; discussion 1893-4.
109. Huxley, R., et al., *Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies.* (0007-0920 (Print)).
110. Giovannucci, E., *Insulin, insulin-like growth factors and colon cancer: a review of the evidence.* (0022-3166 (Print)).
111. Hoyo, C., et al., *IGF2R genetic variants, circulating IGF2 concentrations and colon cancer risk in African Americans and Whites.* Dis Markers, 2012. **32**(2): p. 133-41.
112. Schmidt, F.M., et al., *Inflammatory cytokines in general and central obesity and modulating effects of physical activity.* (1932-6203 (Electronic)).
113. Kopp, H.P., et al., *Impact of weight loss on inflammatory proteins and their association with the insulin resistance syndrome in morbidly obese patients.* (1524-4636 (Electronic)).
114. Pendyala, S., et al., *Diet-induced weight loss reduces colorectal inflammation: implications for colorectal carcinogenesis.* (1938-3207 (Electronic)).
115. Park, H., et al., *A high-fat diet increases angiogenesis, solid tumor growth, and lung metastasis of CT26 colon cancer cells in obesity-resistant BALB/c mice.* Mol Carcinog, 2012. **51**(11): p. 869-80.
116. Park, S.L., et al., *Body size, adult BMI gain and endometrial cancer risk: the multiethnic cohort.* Int J Cancer, 2010. **126**(2): p. 490-9.
117. Crosbie, E.J., et al., *Body mass index, hormone replacement therapy, and endometrial cancer risk: a meta-analysis.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(12): p. 3119-30.
118. Kern, S., Calhoun Eric, *Molecular Genetics of Pancreatic Cancer*. 2008.
119. Eser, S., et al., *Oncogenic KRAS signalling in pancreatic cancer.* (1532-1827 (Electronic)).
120. Maitra, A., R.H. Kern Se Fau - Hruban, and R.H. Hruban, *Molecular pathogenesis of pancreatic cancer.* (1521-6918 (Print)).
121. Hruban, R.H., et al., *K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization.* (0002-9440 (Print)).
122. Lowenfels, A.B., et al., *Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group.* (0028-4793 (Print)).

123. Malka, D., et al., *Risk of pancreatic adenocarcinoma in chronic pancreatitis.* (0017-5749 (Print)).
124. Tang, Y., et al., *An increased abundance of tumor-infiltrating regulatory T cells is correlated with the progression and prognosis of pancreatic ductal adenocarcinoma.* (1932-6203 (Electronic)).
125. Lesina, M., et al., *Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer.* (1878-3686 (Electronic)).
126. Li, M., et al., *Interleukin-8 increases vascular endothelial growth factor and neuropilin expression and stimulates ERK activation in human pancreatic cancer.* (1349-7006 (Electronic)).
127. Matsuo, Y., et al., *CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer.* (1097-0215 (Electronic)).
128. Verma, G., M. Bhatia H Fau - Datta, and M. Datta, *Gene expression profiling and pathway analysis identify the integrin signaling pathway to be altered by IL-1beta in human pancreatic cancer cells: role of JNK.* (1872-7980 (Electronic)).
129. Greco, E., et al., *Pancreatic cancer cells invasiveness is mainly affected by interleukin-1beta not by transforming growth factor-beta1.* (0393-6155 (Print)).
130. Truty, M.J. and R. Urrutia, *Basics of TGF-beta and pancreatic cancer.* (1424-3911 (Electronic)).
131. Chopra, V., E.V. Dinh Tv Fau - Hannigan, and E.V. Hannigan, *Circulating serum levels of cytokines and angiogenic factors in patients with cervical cancer.* (0735-7907 (Print)).
132. Poch, B., et al., *Systemic immune dysfunction in pancreatic cancer patients.* (1435-2443 (Print)).
133. Jacobs, E.J., et al., *Serum transforming growth factor-beta1 and risk of pancreatic cancer in three prospective cohort studies.* (1573-7225 (Electronic)).
134. Omary, M.B., et al., *The pancreatic stellate cell: a star on the rise in pancreatic diseases.* (0021-9738 (Print)).
135. Sideras, K., et al., *Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies.* (1532-1967 (Electronic)).
136. Inman, K.S., A.A. Francis, and N.R. Murray, *Complex role for the immune system in initiation and progression of pancreatic cancer.* (2219-2840 (Electronic)).
137. von Bernstorff, W., et al., *Systemic and local immunosuppression in pancreatic cancer patients.* (1078-0432 (Print)).
138. Bronte, V. and P. Zanovello, *Regulation of immune responses by L-arginine metabolism.* (1474-1733 (Print)).
139. Willimsky, G. and T. Blankenstein, *The adaptive immune response to sporadic cancer.* Immunol Rev, 2007. **220**: p. 102-12.

140. Wang, R.F., *Immune suppression by tumor-specific CD4+ regulatory T-cells in cancer.* Semin Cancer Biol, 2006. **16**(1): p. 73-9.
141. Frey, A.B. and N. Monu, *Signaling defects in anti-tumor T cells.* Immunol Rev, 2008. **222**: p. 192-205.
142. Yamamoto, T., et al., *Circulating CD4+CD25+ regulatory T cells in patients with pancreatic cancer.* (1536-4828 (Electronic)).
143. Shevchenko, I., et al., *Low-dose gemcitabine depletes regulatory T cells and improves survival in the orthotopic Panc02 model of pancreatic cancer.* (1097-0215 (Electronic)).
144. Hiraoka, N., et al., *Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions.* Clin Cancer Res, 2006. **12**(18): p. 5423-34.
145. Ghansah, T., et al., *Dendritic cell immunotherapy combined with gemcitabine chemotherapy enhances survival in a murine model of pancreatic carcinoma.* Cancer Immunol Immunother, 2013. **62**(6): p. 1083-91.
146. Youn, J.I., et al., *Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice.* J Leukoc Biol, 2012. **91**(1): p. 167-81.
147. Rodriguez, P.C. and A.C. Ochoa, *Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives.* (1600-065X (Electronic)).
148. Raber, P.L., et al., *Subpopulations of myeloid-derived suppressor cells impair T cell responses through independent nitric oxide-related pathways.* (1097-0215 (Electronic)).
149. Gehad, A.E., et al., *Nitric oxide-producing myeloid-derived suppressor cells inhibit vascular E-selectin expression in human squamous cell carcinomas.* (1523-1747 (Electronic)).
150. Gorelik, L. and R.A. Flavell, *Transforming growth factor-beta in T-cell biology.* (1474-1733 (Print)).
151. Ding, L. and E.M. Shevach, *IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function.* (0022-1767 (Print)).
152. Nausch, N., et al., *Mononuclear myeloid-derived "suppressor" cells express RAE-1 and activate natural killer cells.* (1528-0020 (Electronic)).
153. Suzuki, E., et al., *Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity.* Clin Cancer Res, 2005. **11**(18): p. 6713-21.
154. Liu, C., et al., *Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host.* (0006-4971 (Print)).
155. Hanson, E.M., et al., *Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells.* (1550-6606 (Electronic)).
156. Solito, S., S. Bronte V Fau - Mandruzzato, and S. Mandruzzato, *Antigen specificity of immune suppression by myeloid-derived suppressor cells.* (1938-3673 (Electronic)).

157. Nagaraj, S., et al., *Antigen-specific CD4(+) T cells regulate function of myeloid-derived suppressor cells in cancer via retrograde MHC class II signaling.* (1538-7445 (Electronic)).
158. Cheng, P., et al., *Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein.* (1540-9538 (Electronic)).
159. Mao, Y., et al., *Inhibition of tumor-derived prostaglandin-e2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity.* (1078-0432 (Print)).
160. Li, H., et al., *Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1.* (1550-6606 (Electronic)).
161. Avalle, L., et al., *STAT1 and STAT3 in tumorigenesis: A matter of balance.* (2162-3988 (Print)).
162. Quinn, M., *The neutrophils respiratory burst oxidase. .* The neutrophils. New outlook for old cells. 2nd ed., 2004: p. 35–85.
163. Movahedi, K., et al., *Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity.* (0006-4971 (Print)).
164. Capietto, A.H., et al., *Down-regulation of PLCgamma2-beta-catenin pathway promotes activation and expansion of myeloid-derived suppressor cells in cancer.* (1540-9538 (Electronic)).
165. Parker, K.H., et al., *HMGB1 enhances immune suppression by facilitating the differentiation and suppressive activity of myeloid-derived suppressor cells.* (1538-7445 (Electronic)).
166. Kujawski, M., et al., *Stat3 mediates myeloid cell-dependent tumor angiogenesis in mice.* (0021-9738 (Print)).
167. Shojaei, F., et al., *Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells.* (1087-0156 (Print)).
168. Oh, K., et al., *A mutual activation loop between breast cancer cells and myeloid-derived suppressor cells facilitates spontaneous metastasis through IL-6 trans-signaling in a murine model.* Breast Cancer Res, 2013. **15**(5): p. R79.
169. Khaled, Y.S., B.J. Ammori, and E. Elkord, *Increased levels of granulocytic myeloid-derived suppressor cells in peripheral blood and tumour tissue of pancreatic cancer patients.* J Immunol Res, 2014. **2014**: p. 879897.
170. Sanford, D.E., et al., *A Study of Zoledronic Acid as Neo-Adjuvant, Perioperative Therapy in Patients with Resectable Pancreatic Ductal Adenocarcinoma.* J Cancer Ther, 2013. **4**(3): p. 797-803.
171. Stromnes, I.M., et al., *Targeted depletion of an MDSC subset unmasks pancreatic ductal adenocarcinoma to adaptive immunity.* (1468-3288 (Electronic)).
172. Vincent, J., et al., *5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity.* Cancer Res, 2010. **70**(8): p. 3052-61.

173. Hursting, S.D., et al., *Reducing the weight of cancer: mechanistic targets for breaking the obesity-carcinogenesis link.* Best Pract Res Clin Endocrinol Metab, 2008. **22**(4): p. 659-69.
174. Osorio-Costa, F., et al., *Epidemiological and molecular mechanisms aspects linking obesity and cancer.* Arq Bras Endocrinol Metabol, 2009. **53**(2): p. 213-26.
175. Bellone, G., et al., *Tumor-Associated Transforming Growth Factor-β and Interleukin-10 Contribute to a Systemic Th2 Immune Phenotype in Pancreatic Carcinoma Patients.* The American Journal of Pathology, 1999. **155**(2): p. 537-547.
176. Corbett, T.H., et al., *Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice.* Cancer Res, 1984. **44**(2): p. 717-26.
177. Turbitt, W.J., et al., *Fish Oil Enhances T Cell Function and Tumor Infiltration and Is Correlated With a Cancer Prevention Effect in HER-2/neu But Not PyMT Transgenic Mice.* Nutr Cancer, 2015. **67**(6): p. 965-75.
178. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
179. Stewart, T.J., et al., *Modulating the expression of IFN regulatory factor 8 alters the protumorigenic behavior of CD11b+Gr-1+ myeloid cells.* (1550-6606 (Electronic)).
180. Gantt, S., et al., *The Role of Myeloid-Derived Suppressor Cells in Immune Ontogeny.* Frontiers in Immunology, 2014. **5**: p. 387.
181. Bierie, B. and H.L. Moses, *Transforming growth factor beta (TGF-beta) and inflammation in cancer.* (1879-0305 (Electronic)).
182. Khaled, Y.S., B.J. Ammori, and E. Elkord, *Increased levels of granulocytic myeloid-derived suppressor cells in peripheral blood and tumour tissue of pancreatic cancer patients.* (2314-7156 (Electronic)).
183. Younos, I., et al., *Tumor- and organ-dependent infiltration by myeloid-derived suppressor cells.* Int Immunopharmacol, 2011. **11**(7): p. 816-26.
184. Shojaei, F., et al., *G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models.* Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6742-7.
185. Yang, L., et al., *Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis.* Cancer Cell, 2008. **13**(1): p. 23-35.
186. Lechner, M.G., A.L. Liebertz Dj Fau - Epstein, and A.L. Epstein, *Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells.* (1550-6606 (Electronic)).
187. Genkinger, J.M., et al., *Central adiposity, obesity during early adulthood, and pancreatic cancer mortality in a pooled analysis of cohort studies. LID - mdv355 [pii].* (1569-8041 (Electronic)).

188. Steele, C.W., et al., *Exploiting inflammation for therapeutic gain in pancreatic cancer.* (1532-1827 (Electronic)).
189. Marti, A., J.A. Marcos A Fau - Martinez, and J.A. Martinez, *Obesity and immune function relationships.* (1467-7881 (Print)).
190. O'Rourke, R.W., et al., *Adipose tissue NK cells manifest an activated phenotype in human obesity.* (1532-8600 (Electronic)).
191. O'Shea, D., et al., *Natural killer cells in obesity: impaired function and increased susceptibility to the effects of cigarette smoke.* (1932-6203 (Electronic)).
192. Leitch, J., et al., *CTL-dependent and -independent antitumor immunity is determined by the tumor not the vaccine.* (0022-1767 (Print)).
193. Waldhauer, I. and A. Steinle, *NK cells and cancer immunosurveillance.* (1476-5594 (Electronic)).
194. Arslan, A.A., et al., *Anthropometric measures, body mass index, and pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan).* Arch Intern Med, 2010. **170**(9): p. 791-802.
195. Renehan, A.G., et al., *Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies.* Lancet, 2008. **371**(9612): p. 569-78.
196. Larsson, S.C., N. Orsini, and A. Wolk, *Body mass index and pancreatic cancer risk: A meta-analysis of prospective studies.* Int J Cancer, 2007. **120**(9): p. 1993-8.
197. Berrington de Gonzalez, A., S. Sweetland, and E. Spencer, *A meta-analysis of obesity and the risk of pancreatic cancer.* Br J Cancer, 2003. **89**(3): p. 519-23.
198. Lashinger, L.M., et al., *Dietary energy balance modulation of Kras- and Ink4a/Arf+/--driven pancreatic cancer: the role of insulin-like growth factor-I.* (1940-6215 (Electronic)).
199. Tseng, W.W., et al., *Development of an orthotopic model of invasive pancreatic cancer in an immunocompetent murine host.* (1078-0432 (Print)).
200. Bayne, L.J., et al., *Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer.* (1878-3686 (Electronic)).
201. Ostrand-Rosenberg, S. and P. Sinha, *Myeloid-derived suppressor cells: linking inflammation and cancer.* (1550-6606 (Electronic)).
202. Sica, A., et al., *Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy.* (0959-8049 (Print)).
203. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* (1471-4906 (Print)).
204. Terabe, M., et al., *Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence.* (0022-1007 (Print)).

205. Farrow, B. and B.M. Evers, *Inflammation and the development of pancreatic cancer.* (0960-7404 (Print)).
206. 9, S., *SEER 9 Incidence and Mortality.*
207. Li, M.O., et al., *Transforming growth factor-beta regulation of immune responses.* (0732-0582 (Print)).
208. Oft, M., *IL-10: master switch from tumor-promoting inflammation to antitumor immunity.* (2326-6074 (Electronic)).
209. Wesolowski, R., J. Markowitz, and W.E. Carson, 3rd, *Myeloid derived suppressor cells - a new therapeutic target in the treatment of cancer.* (2051-1426 (Electronic)).
210. Nefedova, Y., et al., *Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells.* (1538-7445 (Electronic)).
211. Bose, A., et al., *Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination.* (1097-0215 (Electronic)).
212. Bao, B., et al., *The complexities of obesity and diabetes with the development and progression of pancreatic cancer.* Biochim Biophys Acta, 2011. **1815**(2): p. 135-46.
213. Kelesidis, I., T. Kelesidis, and C.S. Mantzoros, *Adiponectin and cancer: a systematic review.* Br J Cancer, 2006. **94**(9): p. 1221-5.
214. Simpson, F. and J.P. Whitehead, *Adiponectin--it's all about the modifications.* Int J Biochem Cell Biol, 2010. **42**(6): p. 785-8.
215. Grote, V.A., et al., *The association of circulating adiponectin levels with pancreatic cancer risk: a study within the prospective EPIC cohort.* (1097-0215 (Electronic)).
216. Pezzilli, R., et al., *Serum leptin, but not adiponectin and receptor for advanced glycation end products, is able to distinguish autoimmune pancreatitis from both chronic pancreatitis and pancreatic neoplasms.* (1502-7708 (Electronic)).
217. Stolzenberg-Solomon, R.Z., et al., *Prediagnostic adiponectin concentrations and pancreatic cancer risk in male smokers.* (1476-6256 (Electronic)).
218. Kadowaki, T., et al., *Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome.* (0021-9738 (Print)).
219. Huang, B., et al., *Adiponectin promotes pancreatic cancer progression by inhibiting apoptosis via the activation of AMPK/Sirt1/PGC-1alpha signaling.* (1949-2553 (Electronic)).
220. Chandran, M., et al., *Adiponectin: more than just another fat cell hormone?* (0149-5992 (Print)).
221. Tsang, J.Y., et al., *Novel immunomodulatory effects of adiponectin on dendritic cell functions.* (1878-1705 (Electronic)).
222. Grossmann, M.E., et al., *Effects of adiponectin on breast cancer cell growth and signaling.* (0007-0920 (Print)).
223. Lovren, F., et al., *Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages.* Am J Physiol Heart Circ Physiol, 2010. **299**(3): p. H656-63.

224. Wolf, A.M., et al., *Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes.* Biochem Biophys Res Commun, 2004. **323**(2): p. 630-5.
225. Lee, Y.A., et al., *Synergy between adiponectin and interleukin-1beta on the expression of interleukin-6, interleukin-8, and cyclooxygenase-2 in fibroblast-like synoviocytes.* (2092-6413 (Electronic)).
226. Javle, M., et al., *Biomarkers of TGF-beta signaling pathway and prognosis of pancreatic cancer.* (1932-6203 (Electronic)).
227. Lu, Z., et al., *Presence of two signaling TGF-beta receptors in human pancreatic cancer correlates with advanced tumor stage.* (0163-2116 (Print)).

VITA

Shawntawnee Collins

EDUCATION

Pennsylvania State University, Ph.D. Immunology and Infectious Disease
2010-2015
Alcorn State University, M.S. Biology
2008-2010
Alcorn State University, B.S. Biology
2004-2008

PUBLICATIONS

Turbitt, W.J., Black, A.J., **Collins, S.D**., Meng, H., Xu, H., Washington, S., Aliaga, C., El-Bayoumy, K., Manni, A., Rogers C. J. Fish oil differentially alters T cell function and tumor growth and induces the emergence of Gr1+/CD11b+ cells in two models of mammary carcinogenesis. Nutrition and Cancer. 2015 Aug-Sep; 67(6):965-75.

Collins, S.D., Turbitt, W.J., Black, A.J., Meng, H., Sosnoki D., Rogers C. J. Gr-1+/CD11b+ myeloid derived suppressor cells inhibit T cell proliferation and augment pancreatic tumor growth *(Manuscript in processs).*

Collins, S.D., Turbitt, W.J., Black, A.J., Meng, H., Sosnoki D., Rogers C. J. Obesity accelerates tumor growth and increases the accumulation of Gr-1+CD11b+ myeloid derived suppressor cells. (*Manuscript in processs)*

AWARDS AND HONORS

-Graduate Student Representative for the Diet and Cancer RIS, 2014
-Winner of MARC travel award, 2014
-Winner of the Diet and Cancer RIS poster competition at the Experimental Biology, San Diego, CA April 2014
-Winner of the Diet and Cancer RIS poster competition at the Experimental Biology Boston, MA April 2013
-Clinical & Translational Science Institute Pre-doctoral Fellow, Penn State University 2012
-Sloan Scholar 2012-2015
-Colorado Chapter of the Penn State Alumni Association Scholarship, 2011
-ASU-PSU Bridges to Doctoral Program, 2008-2010