COMPARING THE EFFECT OF THREE EXERCISE INTERVENTIONS ON PANCREATIC TUMOR GROWTH IN MICE MODEL

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DEDICATION

Dedicated to my husband, daughter and parents.

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ABSTRACT

Pancreatic cancer is a highly lethal disease with only a 9% five-year survival rate. It is the fourth leading cause of cancer-related death in the United States. Pancreatic cancer patients often experience reduced fitness and strength, which limits treatment options and diminishes quality of life. Exercise training yields improvements in physical function and reduces fatigue among pancreatic cancer patients. In well-studied cancers, such as breast cancer, different exercise protocols (that is, training programs differing in exercise duration, frequency, type, and intensity) have been compared and results suggest that longer duration moderate-intensity exercise might improve patient outcomes such as quality of life (QoL). However, no study has yet compared different exercise interventions for people with pancreatic cancer. Thus, identifying an optimal exercise intervention remains a critical area to improve pancreatic patients' outcomes. In animal studies, there is also some evidence that exercise may slow tumor growth. Unfortunately, translation to the clinic has remained limited. This is due in part to the majority of the literature finding a benefit from exercise training when it has been used pre-emptively, not initiated after tumor development. Secondly, exercise-interventions have frequently been compared to no intervention at all, rather than in addition to conventional cancer treatments such as chemotherapy. Third, no study has yet compared the effects of different exercise interventions on pancreatic tumor outcomes. Therefore, in this dissertation we investigated the effect of the three exercise interventions; continuous moderate intensity-Low volume (Cont-LV), continuous moderate intensity-high volume (Cont-HV), and high

intensity interval training (HIIT) on pancreatic tumor growth in mice. Exercise training was initiated after tumor development and was provided alongside chemotherapy for approximately two weeks. Exercise training may enhance immune cell infiltration into tumors as well as immune cell cytotoxicity, potentially explaining slower tumor growth in exercise-trained animals. Therefore, the second aim of this study was to investigate the effects of the three exercise interventions on immune cell tumor infiltration and function. As the vast majority of the existing literature has investigated the effect of exercise on tumor growth without chemotherapy, we also compared the effects of the three exercise interventions on tumor growth and immune cell infiltration and function in mice not receiving gemcitabine. We report no difference in tumor volume between the exercise groups and sedentary mice in the experiments that provided gemcitabine treatment. In contrast, we did find an effect of exercise in tumor-bearing mice not treated with gemcitabine, apparently driven by the tumor-inhibiting effect of continuous low-volume exercise. These effects were accompanied by an increase in the CD8+ T-cells in tumors in the continuous low volume exercise group. Overall, the results suggest that two-week long exercise interventions do not provide further benefit on tumor growth or immune function in pancreatic tumor bearing mice receiving chemotherapy. The results also support that exercise can suppress pancreatic tumor growth, potentially by promoting CD8+ T-cell infiltration in tumors in mice not receiving chemotherapy. Future research should explore the effects of longer exercise interventions as well as the effects of the timing of the exercise intervention on tumor growth and anti-tumor immune activity.

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CHAPTER I

1. INTRODUCTION

Pancreatic cancer is a highly lethal disease with only a 9% five-year survival rate. It is the fourth leading cause of cancer-related death in the United States (Siegel et al., 2019). Pancreatic ductal adenocarcinoma (PDAC) is one of the most common types of pancreatic cancer. Approximately 95% of pancreatic cancer cases are PDAC. Problems associated with pancreatic cancer are multifactorial in nature. People diagnosed with pancreatic cancer are generally older and have overall poor health. There is no precise detection technique to diagnose pancreatic cancer at an early stage. Moreover, PDAC tumors metastasize early in the disease course, limiting the effectiveness of therapies such as surgery and radiation (Oberstein and Olive, 2013). Symptoms related to fatigue, pain and psychological distress are often present at the time of diagnosis and become more severe with the progression of the disease. PDAC patients frequently experience cancer-related fatigue and cachexia with weight loss with a profound negative impact on a patient's quality of life (QoL) (Niels et al., 2018). Together, these observations support that pancreatic cancer is one of the hardest cancers to treat.

In recent years, research efforts have been made to develop new treatment options for pancreatic cancer patients, such as targeting tumor stroma regions to facilitate drug delivery. Along with conventional cancer treatment such as surgery and chemotherapy, some studies have examined the effect of exercise on pancreatic cancer patients' overall health. Exercise training including aerobic and resistance training reduced psychological distress during and after treatment in patients (Cormie, 2014; Ngo-huang et al., 2017;

Niels et al., 2018; Segal et al., 2017). Despite recognizing its benefits, many oncologists still fail to recommend exercise, due in part to concerns of its tolerability as well as lack of knowledge of a precise exercise prescription (Park et al., 2015; Smaradottir et al., 2015). In some exercise oncology studies, such as breast cancer, different exercise protocols (that is, training programs differing in exercise duration, frequency, type, and intensity) have been compared and the results support that longer duration moderate-intensity exercise might improve patient outcomes such as quality of life (QoL) (Anderson et al., 2012; Segal et al., 2017). However, no study has compared different exercise programs for people with pancreatic cancer. Thus, identifying an optimal exercise intervention program remains a critical area to improve pancreatic patients' outcomes.

Considerable heterogeneity exists in the literature regarding exercise training (frequency, intensity, type, and duration) in both human and animal models. These differences in exercise training programs complicate interpretation of the effects of exercise during cancer. Beneficial, null, and negative effects of exercise in animal models of cancer can be found in the literature, potentially because of differences in the exercise intervention selected. Furthermore, animal studies have by-and-large have used voluntary wheel running as the exercise training stimulus. While voluntary exercise allows animals to choose exercise time and intensity and is less stressful (Svensson et al., 2016), it does not allow the comparison of the effects of specific intensities or volumes of exercise. Control of these parameters is provided by forced treadmill exercise and was therefore selected in this current project. The existing studies also differ in the timing or initiation of exercise relative to tumor growth. When exercise training is begun prior to tumor development, tumors either grow more slowly (Bacurau et al., 2007; Uhlenbruck and

Order, 1991) or there is no effect on tumor growth (Woods et al., 1994; Yan and Demars, 2011). Similarly, when the exercise intervention is begun after tumor cell inoculation studies either reported or no (Schadler et al., 2016; Zielinski et al., 2004) or enhanced tumor growth (Schadler et al., 2016) .

While there is some support in the animal literature to suggest the importance of exercise post-diagnosis, they differ from true clinical care settings, as most animal studies have not provided any treatment besides the exercise. Instead, it is important to understand the effect(s) of exercise when provided alongside conventional treatments, including surgery, radiation, chemotherapy, or immunotherapy. We do not yet fully understand if exercise and conventional therapies will interact, although it is conceivable that conventional therapy and exercise will each influence the other. For example, conventional therapies could lower exercise capacity meaning the patient is unable to perform the desired bout of training. Alternatively, exercise could potentially change drug metabolism. So far, only one study has determined the effect of exercise on pancreatic tumor volume concurrent with chemotherapy. In a mouse model of pancreatic cancer, mice inoculated with PDAC4662 tumor cells and treated with a chemotherapeutic gemcitabine also performed moderate treadmill running exercise throughout treatment. Encouragingly, exercised mice showed a significant decrease in tumor volume (Schadler et al., 2016). However, this study only considered the effect of moderate-intensity exercise on tumor volume. It is not known if lower levels of exercise would have a similar effect, or if even greater improvements can be gained from higher intensity exercise. Therefore, this dissertation aimed to assess the effects of three exercise interventions differing in

duration, frequency, and intensity on pancreatic tumor growth in the presence and absence of chemotherapy.

Exercise is likely to improve cancer patient outcomes across several domains, including cardiovascular fitness, skeletal muscle functioning, and immune system function. Exercise-induced improvements in immune function have been proposed to explain the observations of reduced tumor burden across a number of different cancer types (Pedersen et al., 2016; Woods et al., 1994; Zielinski et al., 2004). Specifically, researchers have linked the exercise-induced mobilization of cytotoxic immune cells such as CD8+ T-cells and natural killer (NK) cells into peripheral circulation and their increased function with slower tumor growth (Pedersen et al., 2016; Song et al., 2017). For example, voluntary wheel running increases the number of tumor-infiltrating NK cells in mouse models of solid-tumor cancers (Pedersen et al., 2016). This is important because tumor-infiltrating immune cells are often associated with the destruction of tumor cells and subsequent reduction of tumor burden, and improved clinical prognosis (Boon et al., 1997; Pagès et al., 2005). Importantly, exercise was provided before tumor growth in these studies, meaning that it is unknown if exercise provided after tumor development can alter tumor-specific immune activity.

Immune cells such as cytotoxic CD8+T-cells are well studied in the cancer literature. Numerous studies have shown that T-cells can recognize and eliminate malignant cells; however, T-cells require MHC I expression on the target cell surface for activation. Unfortunately, tumor cells frequently downregulate or loose entirely their MHC I expression and thus avoid T-cell mediated killing. In this scenario, NK cells play a particularly important role in killing tumor cells, as NK cells can recognize and eliminate

the MHC-I deficient cells. Further, NK cells are highly responsive to acute dynamic exercise, being recruited to peripheral blood at greater numbers than other lymphocyte subsets (Timmons and Cieslak, 2008). We and others have shown that NK cell ability to kill tumor cells ex vivo (NK cell Cytotoxic Activity, NKCA) is transiently enhanced by acute aerobic exercise (Bigley et al., 2014; Gupta et al., 2018). Importantly, NKCA appears to be related to exercise intensity, with greater changes appearing with greater exercise intensity (Nieman et al., 1993b). Fewer studies have been conducted to determine the effects of exercise training on immune cell numbers and functions. Moderate exercise training in breast cancer survivors did not yield changes in NK-cell number and function (Nieman et al., 1995c). In contrast, more recent studies reported an increase in NKCA after moderate exercise training among breast cancer survivors (Fairey et al., 2005) and stomach cancer patients (Na et al., 2000). These studies only assessed the effect of moderate exercise training on NK cell number and function. The impact of lower and higher duration and intensities of exercise training on NK cell number and function in pancreatic cancer patients is still unknown.

1.1 Aims and hypotheses

Although exercise training improving quality of life in cancer patients, decrease risk and progression cancer, and enhance anti-tumor immune activity, gaps in the literature preclude the consistent recommendation of exercise by oncologists. Especially little is known about exercise training with regard to pancreatic cancer, and pancreatic cancer remains one of the most deadly. The overarching aim of this dissertation is to compare the effect of three different exercise interventions on tumor growth and anti-

tumor immune activity in a mouse model of pancreatic cancer. To achieve this aim, pancreatic tumor-bearing mice completed three different exercise interventions: continuous moderate intensity -low volume exercise (Cont-LV), continuous-moderate intensity-high volume exercise (Cont-HV), and high intensity interval training exercise (HIIT); sedentary mice acted as controls. Outcomes included tumor growth, number of tumor-infiltrating lymphocytes, and NK cell function. Exercise was administered in animals receiving concomitant gemcitabine (chemotherapy) treatment and in untreated animals.

Specifically, this dissertation tested the following aims:

Aim 1.1.1- Investigate the effects of three different exercise interventions on mouse pancreatic tumor growth with gemcitabine treatment.

Hypothesis 1.1.1.1 – Mice assigned to exercise will have slower tumor growth compared to sedentary mice.

Hypothesis 1.1.1.2 – Exercise of greater intensity and longer duration will yield slower tumor growth, such that tumor growth will be slowest in HIIT, followed by Cont-HV, and then Cont-LV.

Aim 1.1.2- Investigate the effects of three different exercise interventions on the number of lymphocytes (CD8+ T-cells, CD4+ T-cells, and NK cells) in blood and tumor and NK cell function in pancreatic-tumor bearing mice receiving gemcitabine.

Hypothesis 1.1.2.1 - Mice assigned to exercise will have more lymphocytes in blood and tumor compared to sedentary mice.

Hypothesis 1.1.2.2 – Exercise of greater intensity and longer duration will yield larger

numbers of lymphocytes in blood and tumor, such that the largest number of cells will be observed in HIIT, followed by Cont-HV, and then Cont-LV.

Hypothesis 1.1.2.3. - Mice assigned to exercise will have higher NK cell cytotoxicity compared to sedentary mice.

Hypothesis 1.1.2.4 – Exercise of greater intensity and longer duration will yield higher NK cell cytotoxicity, such that the highest NK cell cytotoxicity will be observed in HIIT, followed by Cont-HV, and then Cont-LV.

Exploratory aims

As much of the existing literature has found an effect of exercise when no other intervention (i.e. chemotherapy) was administered, this dissertation will also compare three different exercise interventions in PDAC-4662 tumor-bearing mice not receiving chemotherapy. As this is an exploratory aim, fewer animals will be assigned to these groups.

Aim 1.1.3- Investigate the effects of three different exercise interventions on pancreatic tumor growth without gemcitabine treatment.

Hypothesis 1.1.3.1 – Mice assigned to exercise will have slower tumor growth compared to sedentary mice.

Hypothesis 1.1.3.2 – Exercise of greater intensity and longer duration will yield slower tumor growth, such that tumor growth will be slowest in HIIT, followed by Cont-HV, and then Cont-LV.

Aim 1.1.4- Investigate the effects of three different exercise interventions on the number

of lymphocytes (CD8+T-cells, CD4+T-cells, and NK cells) in blood and tumor and NK cell function in pancreatic-tumor bearing mice without gemcitabine treatment.

Hypothesis 1.1.4.1 - Mice assigned to exercise will have more lymphocytes in blood and tumor compared to sedentary mice.

Hypothesis 1.1.4.2 – Exercise of greater intensity and longer duration will yield larger numbers of lymphocytes in blood and tumor, such that the largest number of cells will be observed in HIIT, followed by Cont-HV, and then Cont-LV.

Hypothesis 1.1.4.3. - Mice assigned to exercise will have higher NK cell cytotoxicity compared to sedentary mice.

Hypothesis 1.1.4.4 – Exercise of greater intensity and longer duration will yield higher NK cell cytotoxicity, such that the highest NK cell cytotoxicity will be observed in HIIT, followed by Cont-HV, and then Cont-LV.

1.2 Assumptions and limitations

This dissertation rests on several assumptions. We assume that the PDAC4662 cells will cause pancreatic tumors in C57Bl/6J mice, and that each mouse would experience cancer progression at a similar rate if no intervention were provided. We further assume that the gemcitabine treatment will slow but not fully abrogate tumor growth. We assume that mice assigned to the exercise groups will be able to complete their assigned exercise. We assume that mice assigned to the sedentary group will not exhibit any more physical activity around their home cage than exercised-mice.

This dissertation is not without limitations. Many different exercise protocols could have been selected for these experiments. The Cont-HV and HIIT exercise

programs were designed based on the American College of Sports Medicine recommendations, and have previously shown tolerability and promise during pancreatic cancer in human (Cormie et al., 2017; Ngo-huang et al., 2017; Niels et al., 2018; Parker et al., 2018) and mice (Schadler et al., 2016). As the Cont-LV and Cont-HV protocols are matched in frequency and intensity, results will suggest if total weekly volume of exercise is an important consideration during moderate intensity exercise. However, HIIT exercise group will differ from the other groups in frequency, duration, and intensity, as we felt it was important to follow recommendations for high-intensity interval training (rather than, for example, increasing frequency to match Cont-LV and Cnnt-HV). We also did not select exercise protocols that would represent greater extremes of training programs, as we wanted to ensure protocols could be completed by the tumor-bearing and gemcitabine-treated mice. We are not considering other type of exercise (such as resistance training) due to the difficulty of administering such exercise programs in mice. A further limitation of this dissertation is that the mice are not running voluntarily. Treadmill running was chosen, as it will allow us to compare specific exercise doses. Forced and voluntary exercise could exert a different effect on behavioral and neural parameters and inflammatory responses that might affect tumor outcomes (Cook et al., 2013; Leasure and Jones, 2008; Sasaki et al., 2016). Another limitation of this study is that we have used only male mice to avoid possible sex effects and make our study comparable to previously published pancreatic study (Schadler et al., 2016). Male and female have different biological properties even on the basic cellular level such as biological response to pain management (Deasy et al., 2007; Du et al., 2004; Ramzan et al., 2020). These differences are also reflected in oncology (Sechzer et al., 1994). It has been shown that

chemotherapeutic toxicity is influenced by sex, where women experience greater cytotoxicity from certain chemotherapeutic drugs than men (Huang et al., 2007).

CHAPTER II

2. LITERATURE REVIEW

This chapter will provide a review of the scientific literature covering pancreatic cancer, physical activity and exercise, and the immune system. Physical activity and exercise describe different concepts; however, they are often confused with one another and are sometimes used interchangeably. Physical activity is defined as body movement produced by skeletal muscles that result in energy expenditure (Caspersen et al., 1985). In daily life, physical activity can be categorized into occupational, sports, conditioning, household, transport, or other activities. Exercise is an important subset of physical activity and relatively easy to quantify. It is defined as a planned, structured, and repetitive activity with a definitive objective, i.e., improvement or maintenance of physical fitness (Caspersen et al., 1985). Exercise prescriptions are often operationalized using the following parameters: Frequency (session per week), intensity (how hard per session), time (session duration), and type (aerobic vs. resistance) (American College of Sports Medicine, 2018). In this document, we will discuss effects of both physical activity and exercise. We will use both terms throughout the document as used in the original literature being referenced.

We begin with an overview of pancreatic cancer and the role that the immune system may play in fighting pancreatic cancer. We then discuss physical activity and exercise and cancer risk as well as the effects of physical activity and exercise during cancer treatment. Much of the literature examining human cancer patients is limited to the effects of physical activity and exercise on quality of life and physical function during

treatment. The effects of physical activity and exercise during cancer treatment on tumor-measures are provided by animal models. Finally, we discuss both acute effects and chronic effects of physical activity and exercise on the immune system.

2.1 Pancreatic Cancer

2.1.1 Overview

Pancreatic cancer is one of the most malignant types of solid tumors. It is the fourth leading cause of cancer–related death in the United States (Siegel et al., 2019). In 2019, around 56,770 people (29,940 men and 26,830 women) were diagnosed with pancreatic cancer, and about 45,750 people (23,800 men and 21,950 women) died of pancreatic cancer in the United States (Siegel et al., 2019). The five-year survival rate is just around 9%, and one-year survival is achieved in less than 20% of cases underscoring the need for novel therapy (Vincent et al., 2011). The absence of detectable clinical symptoms and lack of specific diagnostic markers makes it difficult to detect pancreatic cancer at an early stage. It means most of the patients diagnosed with pancreatic cancer already suffer from advanced stage cancer. For patients with resectable tumors, surgery remains the best therapeutic method. For other patients, neoadjuvant and adjuvant therapies, including gemcitabine and fluorouracil, are used to reduce the size of the primary tumor to improve the chances of surgical intervention (Oberstein and Olive, 2013; Reynolds and Folloder, 2014). The etiology of pancreatic cancer is largely unknown. However, there are established risk factors, including smoking, family history of chronic pancreatitis, advancing age, diabetes mellitus, obesity, a high-fat diet, diets high in meat

and low in vegetables and folate, and possibly Helicobacter pylori infection and periodontal disease (Klein et al., 2004; Vincent et al., 2011).

Pancreatic cancer is classified into two major categories according to the type of cells it originates: exocrine and endocrine tumors. Exocrine pancreatic tumors are further divided into adenocarcinomas and a variety of other pancreatic neoplasms. Pancreatic Ductal Adenocarcinoma (PDAC), a cancer of the exocrine pancreas, is the most frequent type of pancreatic cancer (Warshaw and Fernandez-del Castillo, 1992). Approximately 95% of all pancreatic cancers are PDAC (Hidalgo, 2010). PDAC originates from non-invasive precursor lesions. Three PDAC precursors have been recognized: intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm (MCN), and pancreatic intraepithelial neoplasia (PanIN). PanIN represents the most common PDAC precursor lesion (Hruban et al., 2004). PDAC is characterized by the presence of desmoplasia, inflammatory cell infiltration, and cancer-associated acinar atrophy (CAA) (Longnecker, 1982). A study that analyzed data from a large number of PDAC patients' biopsies by using a mathematical analysis predicted that pancreatic cancer cells acquire the ability to metastasize during early PanIN stages. They also predicted that chemotherapy followed by surgery promotes overall more prolonged survival compared to surgery alone (Haeno et al., 2012).

2.1.2 Pancreatic cancer and immune system

The immune system has a crucial role in both the positive and negative regulation of tumor progression (Disis, 2010; Hanahan and Weinberg, 2011; Janssen et al., 2017). In humans, the immune system is categorized into innate and adaptive immune systems. The

innate immune system, including cells such as monocytes, dendritic cells, and natural killer (NK) cells, provides a first line of defense against infectious agents and tumor cells. The adaptive immune system is slower to protect than the innate system but provides a more specific and targeted response. Adaptive responses are mediated in part by B cells and CD4+ and CD8+ T-cells. Once activated, these recognize and eliminate infected and malignant cells.

CD8+ T-cells are the predominant T-cell subset of tumor-infiltrating lymphocytes (TIL) in many types of cancer, including pancreatic cancer (Emmrich et al., 1998). Upon activation by innate antigen-presenting cells, CD8+ T-cells differentiate into cytotoxic T lymphocytes (CTLs) and kill tumor cells by releasing perforin and granzyme (Zhang and Bevan, 2011). Type 1 CD4+ T helper cells (Th-1) also exhibit anti-tumoral responses through secretion of high amounts of pro-inflammatory (Th-1) cytokines such as IL-2, TNF-α, and IFN-γ. These promote not only CD8+ T-cell mediated killing but also increase the presentation of tumor antigen and anti-tumoral activity of macrophages and NK cells (Chang et al., 2016; Pardoll and Topalian, 1998; Zhu and Paul, 2008). The presence of tumor-infiltrating CD4+ and CD8+ T-cells and Th-1 cytokines in tumors correlate with a favorable prognosis in terms of overall survival and disease-free survival in pancreatic cancer (Fukunaga et al., 2004).

Unfortunately, similar to other cancer cells, pancreatic cancer cells have strategies to avoid immune-mediated cytotoxicity (Chang et al., 2016) and thus promote tumor progression (Dunn et al., 2002). While intraepithelial CD8+ T-cells infiltrate is present in low-grade premalignant pancreatic lesions, infiltration is reduced with disease progression (Hiraoka et al., 2006). Further, pancreatic patients exhibit a decrease in the percentage of

circulating CD4+ and CD8+ T-cells (Bang et al., 2006; Xu et al., 2014). Activation of CD8+ T-cells requires presentation of tumor-specific antigens on MHC I molecules. Tumor antigens are derived from mutations in cellular genes that are generally absent in healthy cells but are expressed by cancer cells. Tumor cells can escape T-cells by acquiring further mutations or by secreting immunosuppressive cytokines (Lind et al., 2004; Pasche, 2001). Further, many tumor cells contain mutations decreasing MHC I expression to avoid recognition. Pancreatic carcinomas are frequently characterized by a total or partial loss of expression in MHC class I antigen, which leads to immune escape (Ryschich et al., 2004).

Natural killer cells are an innate lymphocyte that may play a special role in fighting tumor cells. Whereas MHC class I molecule expression is required for T-cell activity, these molecules act as ligands for inhibitory receptors of NK cells, thereby preventing NK cells from damaging normal healthy cells. Loss of MHC class I expression lowers the inhibitory signal for NK cells. As a result, activating receptors on NK cells can recognize and eliminate tumor cells directly through NK cell-mediated cytotoxicity, i.e., exocytosis of perforin-containing secretory lysosome (Caligiuri, 2008; Lanier, 2005; Long et al., 2013; Pegram et al., 2011; Vivier et al., 2008). NK cells also trigger apoptotic pathways in tumor cells through the production of TNFα or via direct cell-cell contact through activation of the TRAIL and FASL pathways (Eischen and Leibson, 1997; Kayagaki et al., 1999; Lind et al., 2004; Takeda et al., 2001; Wallin et al., 2003; Wang et al., 2012; Zamai et al., 1998). In addition to their cytotoxic function, NK cells also secrete chemokines and cytokines, such as TNF-α and IFN-γ, recruit other immune cells and influence adaptive immunity (Hall et al., 2010; Malhotra and Shanker, 2011). As NK cells

can kill pancreatic tumor cells lacking MHC class I, it seems they might play a role in pancreatic cancer treatment. Indeed, several studies have reported a significant increase in the percentage of circulating NK cells in pancreatic cancer patients compared to healthy subjects (Ullenhag et al., 2017; Xu et al., 2014) although other studies have found no significant differences (Bang et al., 2006; Poch et al., 2007). It may be that pancreatic tumors have additional mechanisms of escape that impact NK cell function. NK cell cytotoxic activity (NKCA) is substantially reduced in pancreatic cancer patients when compared with NK cells from sex- and age-matched healthy controls (Bang et al., 2006). This reduction is probably due to decreased production of perforin and granzyme B, both on mRNA and protein levels (Baine et al., 2011; Peng et al., 2014, 2013). Pancreatic cancer also appears to reduce NK cell capacity to secrete immune-activating cytokines such as TNF-α and IFN-γ and decreases the expression of activation receptors such as NCRs (NKp30, and NKp46) and NKG2D (Peng et al., 2014, 2013). This reduction in the activating receptor on NK cells is associated with disease progression (Peng et al., 2013). In support of this work, Funa et al. showed that both basal NK cell activity and *in vitro* responses of NK cells toward IFN-α (an activating cytokine) were reduced in pancreatic patients (Funa et al., 1984). In some cases, chemotherapy may rescue NK cell function. The chemotherapeutic drug gemcitabine was demonstrated to enhance expression of the MICA/B ligand that sensitizes PDAC tumor cells to NK-cell killing (Xu et al., 2011).

2.2 Physical activity, exercise, and cancer

2.2.1 Physical activity/exercise and risk of cancer

An active lifestyle is protective against cancer mortality (Wiseman, 2008). The evidence that physical activity and/or lifelong exercise protect against colon, colorectal, and breast (diagnosed postmenopausal) cancers is conv incing. Evidence for physical activity protecting against other cancers, including pancreatic, is rated as 'limited' suggestive (Kruk and Czerniak, 2013). Nonetheless, approximately twenty studies have analyzed the effect of physical activity on pancreatic cancer risk in humans; these studies report the average risk reduction of pancreatic cancer by physical activity is between 40-50% (Kruk and Czerniak, 2013).

2.2.2 Physical activity/exercise during cancer treatment

Multiple studies have demonstrated that exercise is safe and can be used as part of cancer care (Granger et al., 2011; Loughney et al., 2015; Parker et al., 2018). Cancer patients often experience cachexia, psychological stress, depression and anxiety. Physical activity and exercise-training appear to reduce these effects, thereby improving quality of life and reducing cancer-related fatigue during and after treatment (Brown and Ligibel, 2017; Hwang et al., 2012; Mishra et al., 2012; Speck et al., 2010). These improvements may be due to increased psychological functioning, cardiovascular fitness, skeletal muscle functioning, the vascular system, and the immune system (Brown et al., 2012; LaVoy et al., 2016). The effects of physical activity and exercise in human patients is largely limited to understanding the impact on quality of life and physical function and is the focus of the

following subsection. We later discuss evidence of vascular and immunological effects during cancer from animal models.

2.2.3 Physical activity/exercise during cancer treatment: Quality of life and physical function (Human studies)

Physical fitness may be an important predictor of outcomes even before cancer treatment is begun. Exercise prehabilitation programs have been shown to improve physical and psychological health outcomes, reduce surgical complications, treatment-related morbidity, hospital lengths of stay, hospital readmissions, and overall health care costs (Cabilan et al., 2015; Silver, 2014; Silver and Baima, 2013; Singh et al., 2013; West et al., 2015). In pancreatic cancer patients, it has been shown that low physical fitness correlates with poor outcomes. Chandrabalan et al. found that patients with a lower anaerobic threshold were more likely to have a prolonged postoperative stay in the hospital, post-operative pancreatic fistula, intra-abdominal abscesses, and less likely to receive adjuvant therapy (Chandrabalan et al., 2013). Improving fitness through exercise prehabilitation programs could therefore improve outcomes for pancreatic cancer patients.

Evidence continues to accumulate that aerobic and resistance exercise can improve various aspects of the lives of cancer patients during treatment. Exercise can reduce symptoms and side effects of conventional cancer treatment and retards the rate at which physiologic systems are affected (Schmitz et al., 2010). Numerous studies have examined the effect of exercise during adjuvant cancer treatment in women with breast cancer. In 1983, Winningham first described the beneficial effects of exercise in cancer patients during chemotherapy treatment. Thirty-minutes of cycling three times per week at 60-85%

maximum heart rate (HRmax) substantially increased physical performance in stage II breast cancer patients undergoing chemotherapy treatment (Winningham, 1983). Other studies have also shown that exercise training during treatment can increase VO_2max and improve cardiopulmonary function in breast cancer patients (Hornsby et al., 2014; Rao et al., 2012). A recent review of 17 studies involving 1,175 breast cancer patients undergoing active cancer therapy found that both aerobic and resistance exercise alone or interventions combining aerobic and resistance exercise are safe and well-tolerated (Fairman et al., 2017). Altogether, these studies suggest that exercise can attenuate many of the adverse effects accompanying treatment, and yield significant, clinically meaningful improvements in fitness and quality of life (QoL).

Compared to other cancer types, the effect of exercise during pancreatic cancer is not as well studied, but there is data to suggest positive outcomes. A study including 20 pancreatic patients undergoing pre-operative cancer treatment demonstrated that 120 minutes of moderate-intensity aerobic and resistance home-based combination exercise training (walking 20 minute per day for at least 3 days per week and strengthening exercise for 30 minute per day for at least 2 days per week) is safe and feasible during treatment but there was wide variability in patient-reported adherence to specific exercise protocols (Ngo-huang et al., 2017; Parker et al., 2018). Home-based low-intensity resistance exercise was shown to be feasible and safe in 30 newly diagnosed advanced older pancreatic patients (median age ~75 years) receiving chemotherapy treatment (Naito et al., 2019). The intervention increased outdoor activity in more than 50% of patients and was positively associated with the global quality of life (Mouri et al., 2018). A larger study of 65-pancreatic cancer patients reported that 6 months of either supervised or home-based progressive resistance exercise is feasible

for pancreatic cancer patients and significantly improved muscle strength, although supervised resistance training is more effective compared to home-based training (Steindorf et al., 2019; Wiskemann et al., 2019). A case study has assessed the effect of high-intensity exercise on pancreatic cancer patient health. High intensity aerobic and resistance exercise (both 70-80% of HRmax) was found to be feasible and safe during pancreatic cancer treatment and improved physical and psychological variables of a Stage IV pancreatic cancer patient (Niels et al., 2018).

Despite the growing base of evidence in support of exercise during treatment, many oncologists still fail to recommend exercise, due in part to concerns of its tolerability and the lack of a specific exercise program for cancer patients undergoing treatment (Park et al., 2015; Smaradottir et al., 2015). Few studies have directly compared different exercise interventions on cancer patient health. Courneya and colleagues have begun to address this issue and compared the three different exercise interventions in breast cancer patients receiving chemotherapy treatment: a standard exercise group (aerobic exercise 25-30 min/day for 5 days/week), a high exercise group (aerobic exercise 50-60 min/session for 3 days/week), and a combined exercise (50-60 minute of aerobic and resistance training per session for 3 days/week). Exercise was performed at 65-70% VO_2peak in all groups. All exercise groups improved overall physical functioning, while the combined exercise was superior to standard exercise-dose for endocrine symptoms and superior to standard and high exercise groups for muscular strength. High exercise was superior to combined dose for aerobic fitness. Both high and combined exercise were effective in patients with clinical levels of depressive symptoms at baseline, and high-exercise led to the greatest improvements in sleep quality (Courneya et al., 2014a, 2014b). Brown and colleagues also

compared low (150 minute per week) and high (300 minute per week) volumes of aerobic exercise at 50-70% of age-predicted maximum heart rate on colon cancer survivors and reported high volume of aerobic exercise yielded the largest improvements in health related quality of life outcomes (Brown et al., 2018a, 2018b, 2016). However, no study has yet compared different exercise interventions on patient outcomes during pancreatic cancer.

2.2.4 Physical activity/exercise during cancer treatment: Tumor growth (Animal studies)

Human studies are primarily focused on physical and psychological outcomes, whereas animal studies can assess the effects of exercise directly on tumor growth. And yet, the animal literature is also mixed as to the effects of exercise training during cancer. This is due in part to heterogeneity in all aspects of study methodology, including the type of tumor, type of animal, outcome measurements, and exercise training regimen. A systematic review of 53 cancer studies using rodent models evaluated tumor outcomes (e.g. tumor incidence, tumor growth, and tumor metastasis), and report the current literature is afflicted by heterogeneity in all aspects of study design, including modality (forced vs. voluntary), exercise-dose response (FITT), and schedule exercise-timing relative to tumor development (Ashcraft et al., 2016).

As exercise at different intensities yields different physiological and gene expression adaptation in mammals (Sasso et al., 2015), it is perhaps not surprising that direct comparisons of different exercise intensities differently impact tumor growth. Colbert and colleagues compared two treadmill protocols (moderate: 20 meters/minute vs. vigorous: 24 meters/minute, each for 45 minutes, 5days/week on 5% grade) in transgenic

$p53^{+/-}$ MMTV-Wnt-1 mice model and reported an increased tumor growth in both groups compared to sedentary (Colbert et al., 2009). In contrast, Almeida and colleagues compared two different intensity swimming protocols (50% and 80% of maximal workload) and reported that the 50% protocol inhibited mammary adenocarcinoma tumor growth, but no change in tumor growth with the 80% protocol (Almeida et al., 2009). Woods and colleagues also compared 'moderate' (18 meters/minute for 30 minute on 5% grade) and 'exhaustive' (18 meters/minute for 30 minute on 5% grade and then speed increased 3 meters/minute in every 30 minutes until exhaustion (usually ends in 2-3 hour)) intensity exercise protocol using forced treadmill and reported no difference in the mammary adenocarcinoma tumor growth and overall tumor incident (Woods et al., 1994). More recently, Malicka and colleagues reported a significant decrease in mammary tumor number among exercise groups relative to sedentary mice and a trend for an inverse relationship between exercise intensity and tumor volume (exercise varied progressively adjusting the speed of the treadmill (low: 0.48–1.34 km/ h, moderate: 0.6–1.68 km/h, high: 0.72–2.0 km/h)) (Malicka et al., 2015).

An important consideration in animal studies is the exercise timing relative to tumor development: that is, do results differ when exercise training is begun before or after tumor formation? A few studies have been conducted to directly compare the effect of exercise timing relative to tumor development. Pederson and colleagues reported that voluntary exercise initiated prior to tumor cell inoculation significantly inhibits fast growing melanoma tumor growth compared to sedentary. However, no difference in tumor growth was reported when exercise was initiated after tumor cell inoculation (Pedersen et al., 2016). In contrast, initiating exercise after tumor cell inoculation in slow

growing DEN-induced and Tg (Grm1) Epv tumor models sufficiently controlled tumor incidence and progression (Pedersen et al., 2016). Shalamzari and colleagues have also compared the effect of timing of exercise training relative to tumor inoculation by testing four conditions: exercise-tumor-exercise, exercise-tumor-rest, rest-tumor-exercise, and rest-tumor-rest. They reported that mammary tumor growth rate is highest in rest-tumor-rest, followed by rest-tumor-exercise and exercise-tumor-rest. Mice in the exercise-tumor-exercise group had the slowest tumor growth (Shalamzari et al., 2014). Schadler and colleagues reported either no effect (pancreatic cancer) or increased tumor volume (melanoma) when exercise is initiated after tumor development (Schadler et al., 2016).

The majority of animal studies have assessed the effects of exercise on cancer in isolation, without considering the interaction effect between exercise and conventional cancer treatments such as surgery, radiation, chemotherapy and immunotherapy. In a 'real world' clinical scenario, exercise would not be a replacement for conventional treatment, but would be used as adjuvant therapy. Therefore, understanding the effect of exercise with currently used cancer treatments is required to better design clinical studies and provides recommendations for cancer patients. The few studies that have examined the effects of exercise provided concurrently with chemotherapy suggest that this combined treatment can reduce tumor growth relative to chemotherapy alone (Betof et al., 2015; Schadler et al., 2016). Betof and colleagues reported that voluntary wheel running initiated immediately after tumor cell inoculation with chemotherapy significantly inhibits tumor growth relative to groups that received no treatment, exercise alone or chemotherapy alone. Further exercise alone also significantly inhibits tumor group relative to groups that received no treatment (Betof et al., 2015). Schadler and colleagues reported

reduced pancreatic tumor volume in mice that received moderate aerobic exercise training alongside chemotherapy, compared with the chemotherapy-only control (Schadler et al., 2016). It is intriguing to imagine if similar effects would be observed with other exercise protocols, and this question is the focus of the current project.

2.3 Physical activity, exercise, and the immune system

Cancer treatments such as chemotherapy, chemoradiation, and surgery frequently cause nausea, vomiting, inflammation, depression, reduced bone mineral density, cardiac toxicity, skeletal muscle wasting, oxidative stress, and mitochondrial damage (Gorini et al., 2018; Nurgali et al., 2018; Pearce et al., 2017). All of these side effects exacerbate fatigue and reduce stamina. Exercise during cancer treatment may improve patient outcomes by countering these negative side effects via improving cardiovascular fitness, muscular strength, and regulating the immune system. By improving physical and psychological functioning, patients may better be able to handle higher doses of treatment, and thus experience a greater chance of eradicating tumor cells.

2.3.1 Effects of acute exercise on immune function

Exercise is well known to transiently mobilize immune cells into peripheral circulation. Exercise-induced shear stress and hormone release (catecholamines and glucocorticoids) cause the detachment of leukocytes from the hepatic, pulmonary, splenic, and vascular reservoirs and move the cells into the peripheral blood compartment (Dhabhar et al., 2012; Dimitrov et al., 2010). This causes a dramatic increase in the number of immune cells including lymphocytes (lymphocytosis) found in the blood during and immediately

after exercise; the magnitude of lymphocytosis is dependent on exercise intensity and duration (Walsh et al., 2011). This lymphocytosis is not uniform, as cell subtypes that have greater cytotoxicity are mobilized preferentially into the periphery with exercise (Bigley et al., 2014; Campbell et al., 2009; Gupta et al., 2018; Simpson et al., 2007). Within lymphocytes, NK-cells are the most exercise-responsive cell, then CD8 T-cells and CD4 T-cells (Timmons and Cieslak, 2008). While high-intensity exercise mobilizes more NK-cells into circulation compared to moderate-intensity exercise, the magnitude of the increase in NK-cells may decrease with the duration of the exercise bout (Shephard and Shek, 1999).

Upon exercise cessation, lymphocyte number quickly returns to baseline, or in the case of vigorous/exhausting exercise, may drop below baseline values (lymphocytopenia). Lymphocyte frequency usually returns to pre-exercise levels within 24 hours (Campbell and Turner, 2018; Simpson et al., 2015). However, high intensity, high volume exercise may cause suppression of immune function for a longer period of time that lasts up to 72 hours or more (Pedersen and Ullum, 1994). If vigorous exercise is repeatedly performed without adequate rest, then the exercise-induced enhancement in immunity is blunted, and the post-exercise immune depression is more severe and prolonged, leaving the individual more susceptible to infection. Thus, proper consideration of exercise intensity and timing (i.e. an exercise training protocol) is critical for the cancer patient.

Acute exercise not only increases the number of NK cells in peripheral circulation, but also enhances their ability to kill tumor cells (Bigley et al., 2014; Gupta et al., 2018). NK cell cytotoxic activity (NKCA) is increased immediately following acute exercise and persists into recovery. This effect is related to exercise intensity, where high-intensity exercise enhances NKCA more than moderate-intensity exercise (Nieman et al.,

1993b). Initially it was thought that the increased NK cell number by exercise explained enhanced killing capacity, and later it was suggested that specific changes in the proportions of NK cell subsets by exercise (specifically, an increase in highly cytotoxic subsets) were primarily responsible for increased NKCA. In support of this later hypothesis, adjusting NKCA by the number of NK cells in blood (NKCA/cell) demonstrates that NK cells present in the blood during the recovery phase of exercise are more cytotoxic against lymphoma and multiple myeloma cell lines *in vitro*. This increase in NK cell function could potentially be explained by increased expression of activating receptors such as NKG2C on NK cells (Bigley et al., 2014). We have also shown that changes in the hormones and cytokines in blood during recovery phase of exercise can enhance NKCA (Gupta et al., 2018).

2.3.2 Effects of chronic exercise on immune function

Numerous studies have been conducted to determine the acute effect of single sessions of exercise on immune cells. However, fewer studies have determined the chronic effect of exercise training on immune cell number and function. The numbers of total leukocytes and leukocyte subsets are similar in athletes and age-matched healthy controls (Nieman et al., 1995a, 1995b). However, longitudinal training studies have reported a decrease in the number of NK cells and CD8 T-cells after 12 weeks and 6 months of intensive exercise training in swimmers and cyclists, respectively (Baj et al., 1994; Gleeson et al., 2000). In ageing adults, it appears that physical fitness can protect against the accumulation of highly differentiated T-cells. Highly differentiated T-cells are unable to respond to novel pathogens and have been associated with a chronic low-grade inflammation. Spielmann et al. showed that across a range of ages (18-61 years), aerobic

fitness (greater VO_2max) was inversely associated with the proportion of high differentiated KLRG1+CD57+ and KLRG1+CD28- CD4+ and CD8+ T-cells present in peripheral blood (Spielmann et al., 2011). Further, the relationship between increasing age and increased proportion of these highly differentiated T-cells was abrogated following adjustments for VO_2max (Spielmann et al., 2011). These results suggest that physical fitness lessens the impact of age on the accretion of highly differentiated T-cells, at least up to a certain age. More recently, similar results were found in an older population (65-85 years), where adults self-reporting either an intense training (>5 days/week of volleyball, basketball, or running for at least five years) or moderate training (2-3 days/week) lifestyle had fewer highly differentiated (CD45RA+CCR7-) T-cells and longer telomere lengths among peripheral T-cells (Silva et al., 2016). Significant inverse correlations between self-reported measure of exercise (especially minutes/month) and senescent p16INKa4+ T-cells have also been found (Liu et al., 2009). There is also evidence that physical fitness protects T-cell function during aging, such as T-cell proliferation (Nieman et al., 1993a) and production of both pro- and anti-inflammatory cytokines (Shinkai et al., 1995) in response to stimulation.

Exercise training is also known to alter NK cell function. Nieman and colleagues reported that highly fit older women have greater baseline NKCA compared to sedentary older women (Nieman et al., 1993a). Woods and colleagues reported a trend for improvement in NKCA against HLA-depleted tumor cells compared to age-matched controls (this did not reach statistical significance) after a 6-month aerobic exercise intervention in older adults (65 years), (Woods et al., 1999). However, other studies report no change in NKCA following 12 months of mixed aerobic and resistance training (age range 50-75 years) (Campbell et al., 2008), or after 12 weeks of aerobic training (mean age 73 years) (Nieman et

al., 1993a). One study reports a decrease in NKCA after a month-long heavy exercise training in college female volleyball players (Suzui et al., 2004).

A few research groups have also studied the effects of exercise training on immune-function amongst cancer patients. Some have reported an improvement in NKCA after exercise training among postmenopausal breast cancer survivors (Fairey et al., 2005) and stomach cancer patients who had undergone curative resection (Na et al., 2000). However, others have reported no change in NK-cell number and function after eight weeks of moderate exercise training (60 minute of supervised weight training and aerobic training three times in a week) in breast cancer patients (Nieman et al., 1995c). In mice, six-weeks of voluntary wheel running significantly increased NK-cell infiltration into the melanoma tumor, although no differences in NKCA were found (Pedersen et al., 2016). Moreover, this increase in NK-cell infiltration was dependent on the concurrent exercise-induced mobilization of immune cells and was shown to be caused by exercise-induced increases in epinephrine and IL-6 (Pedersen et al., 2016). An increase in NK cell number and function has been reported in breast cancer obese mice after high intensity interval training on a treadmill (Barra et al., 2017). While the effects of acute and chronic exercise in healthy adults are well documented, whether exercise training impacts NK-cell number and function amongst pancreatic cancer patients or in animal models of pancreatic cancer needs to be determined.

2.4 Summary

Exercise is beneficial for human beings as it improves mental and physical health. Exercise not only has a protective effect against diseases such as diabetes and

cardiorespiratory disease but it is also helpful in mitigating the adverse effects generated by these diseases. In recent years, several studies have explored the effect of exercise on cancer patients. Numerous studies have been conducted on breast cancer patients and report that exercise reduces stress and anxiety and thus results in improved patients reported outcomes. Animal studies can help us assess the direct effect of exercise on tumor burden. However, outcomes in the current literature are mixed, with a few studies reporting increased tumor growth and others report decreased or no effect on tumor growth. These different outcomes may partly be explained by differences in the exercise interventions, which varied by frequency, intensity, type, duration, whether exercise was voluntary or forced, and the timing of when exercise was initiated relative to tumor cell inoculation. Collectively, these differences make it difficult to compare the effects of exercise training on cancer outcomes across different studies. Therefore, this dissertation aimed to examine the effects of three different exercise interventions on pancreatic tumor growth. To make our study more relevant to a clinical setting, exercise was initiated after tumor development and was offered concurrently with gemcitabine.

Exercise is known to mobilize immune cells in peripheral circulation, particularly highly differentiated cytotoxic lymphocytes, and enhances immune function. NK cells in particularly are highly responsive to exercise, with large increases in NK cell number in circulation immediately after exercise and increases in NKCA. Pancreatic cancer patients have a reduced number of circulating lymphocytes (CD4 T-cells, CD8 T-cell, and NK cells) compared to healthy adults. If exercise can yield similar improvements in immune function in these patients as observed in healthy adults, it may prove to be a adjuvant therapy to improve immune cell functions. Importantly, NK cell function after exercise is enhanced in

breast cancer patients. Further, animal studies show exercise enhanced NK cell infiltration in tumors.

In summary, existing literature supports the fact that exercise is beneficial for cancer patients undergoing treatment. However, it is not know what is optimal exercise intervention for patients in terms of frequency, intensity, type, and duration. To address this issue, here we compared the effect of three different exercise interventions on tumor growth, lymphocytes number (T and NK cells) and NK cells functions.

CHAPTER III

3. MATERIALS AND METHODS

3.1 Mice and animal care

Six-week-old M. musculus C57BL/6 mice were used for this study. Only male mice were used in this study to avoid possible effects arising from female sex hormones. A total of 84 male C57BL/6J mice were purchased from Jackson Laboratories. Mice were purchased at three different time points. Initially, 30 mice were purchased for the first phase of experiments. Later, 30 and 24 mice were purchased for a second and third phase of experiments, respectively. Prior to starting the experiment, all the required documents and forms were submitted to the MD Anderson Institutional Animal Care and Use Committee; experiments began after approval was granted. Mice were maintained in standard housing cages in a thermo-stated environment under a 12h light/dark cycle with free access to food (normal chow, 2844kcal/kg, 4% crude fat) and drinking water throughout the experiment. Mice were acclimated to the facility at MD Anderson for at least 48 h before the start of the experiment. Mice were examined on a regular basis.

3.2 Experimental Design

Table 3.1 summarizes the groups of mice that were used in these experiments. In total, 84 mice were used in the experiments. All animals were inoculated with PDAC4662 cells at the start of the experiment to induce pancreatic cancer. Mice were assigned to their respective intervention group once tumors presented (~55mm^3, approximately 7 days post-inoculation). Group assignments were made to ensure that groups were

approximately equal with regard to tumor volume at the start of the intervention (Replication 1: $p = 0.99$; Replication 2: $p = 0.988$). To answer Aims 1 and 2, 60 mice were randomly distributed into 5 groups: sedentary (Sed), sedentary with gemcitabine (Sed+Gem), continuous moderate intensity-low volume exercise training with gemcitabine (Cont-LV+Gem), continuous moderate intensity-high volume exercise training with gemcitabine (Cont-HV+Gem), high intensity interval training with gemcitabine (HIIT+Gem). Animals in the Sed group were used to ensure that the PDAC4662 cells induce pancreatic tumors. Thirty mice (6 animals per group) were used in two identical experiments performed successively to assess the experimental variability. Tumor volume was measured every other day. To analyze the effect of exercise without chemotherapy treatment on pancreatic tumor outcomes (Exploratory Aims: Aims 3 and 4), 24 mice were distributed in 4 groups (Sed, Cont-LV, Cont-HV, HIIT). These experiments were identical to those described for Aims 1 and 2 except that animals did not receive gemcitabine. Mice were euthanized after two weeks of exercise intervention or earlier due to ulcer development. Blood, spleen and tumor were collected for outcome measures. Figures 1 and 2 describe the experiments to answer Aims 1 and 2, and 3 and 4, respectively.

Table 3-1: Description of groups involved in this study

Groups		Without chemotherapy	With chemotherapy (Gemcitabine Drug)
Aims 1-2	No exercise	Sed (N=6*2)	Sed+Gem (N=6*2)
	Continuous moderate intensity-Low volume	-	Cont-LV+Gem (N=6*2)
	Continuous moderate intensity--High volume	-	Cont-HV+Gem (N=6*2)
	High intensity Interval training	-	HIIT+Gem (N=6*2)
Aims 3-4	No exercise	Sed (N=6)	-
	Continuous moderate intensity Low volume	Cont-LV (N=6)	-
	Continuous moderate intensity-High volume	Cont-HV (N=6)	-
	High intensity Interval training	HIIT (N=6)	-

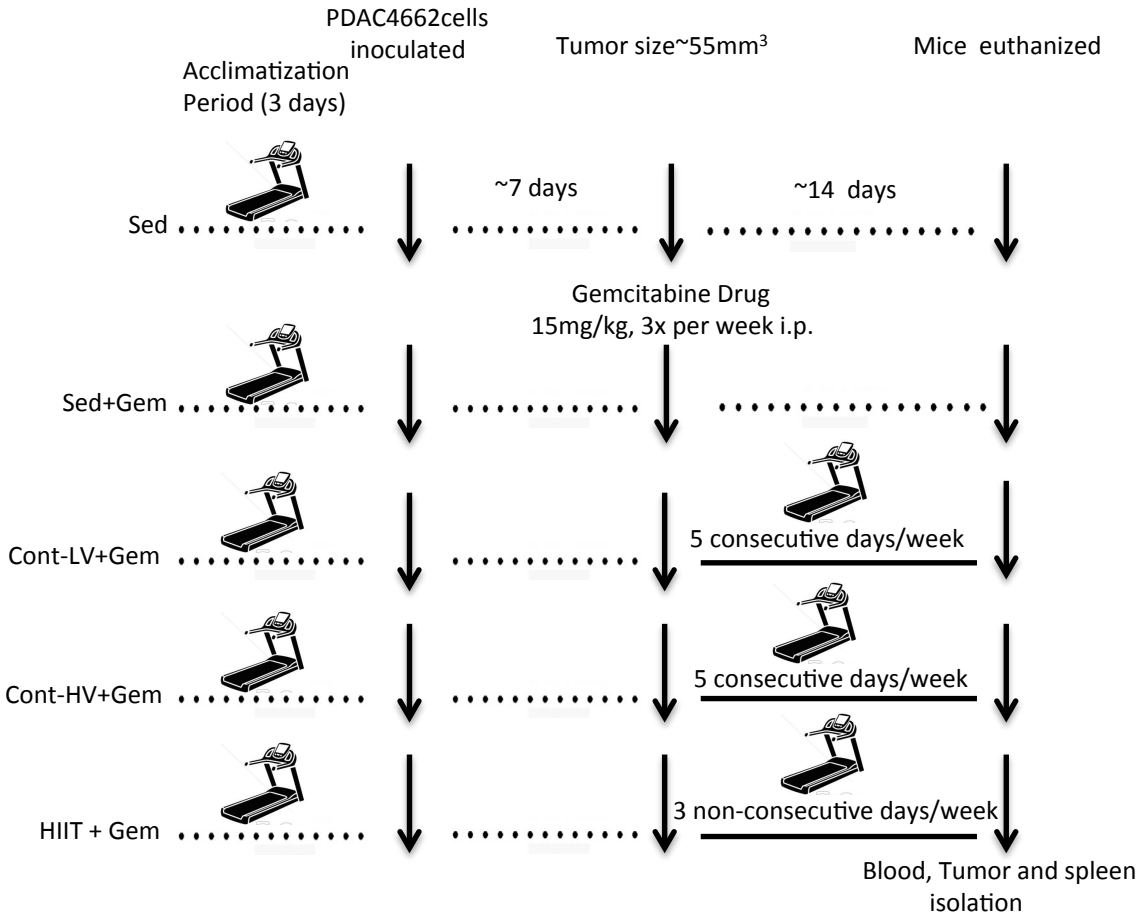


Figure 3.1. Experimental design for Aim1 and 2 - Treatment (exercise and/or gemcitabine, where applicable) was given when tumors were palpable and were continued until euthanasia. Tumor volume was measured throughout the experiment

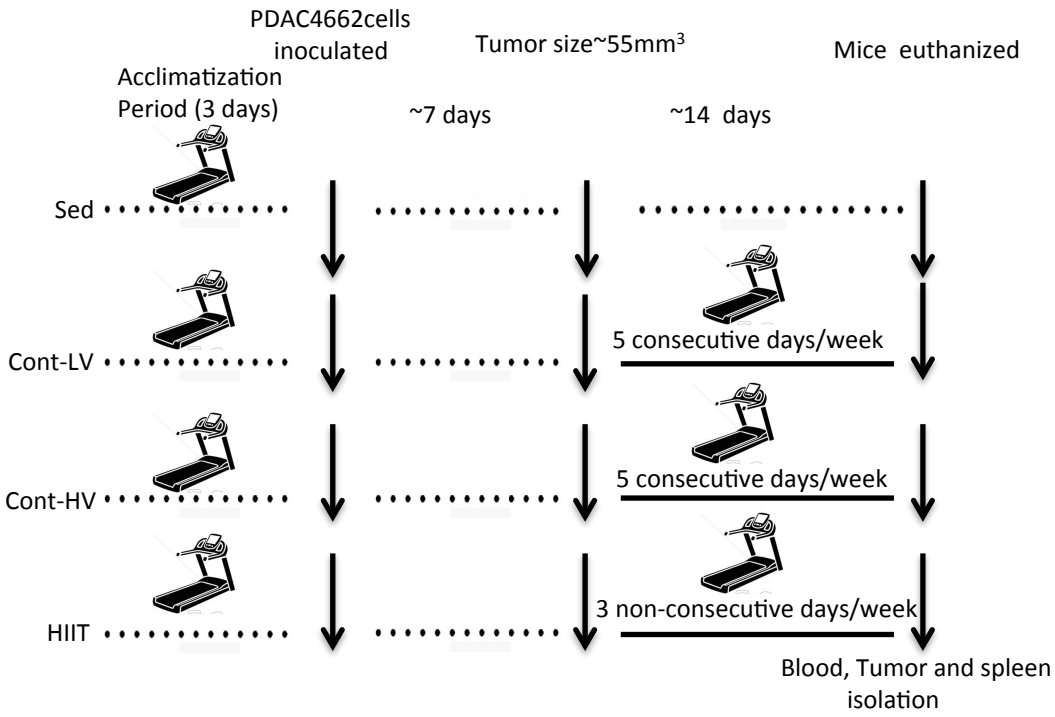


Figure 3.2. Experimental design for Aim 3 and 4 - Treatment (exercise where applicable) was given when tumors were palpable and were continue until euthanasia. Tumor volume was measured throughout the experiment

3.3 PDAC4662 cell line maintenance and inoculation

PDAC4662 cell lines were originally generated from KrasLSL-G12D/+, Trp53LSL-R172H/+, Pdx1-Cre (KPC) mice bred in-house. Dr. Robert Vonderheide (Professor at the University of Pennsylvania School of Medicine) provided these cells for these experiments and was grown in Dr. Schadler's laboratory at MD Anderson. The cell lines were preserved in a protein-free, sterile cryopreservation medium containing 10% DMSO in cryovials (USA Scientific, Ocala, FL) in liquid nitrogen. A week before inoculation, cell lines were thawed and grown in glutamine- enriched, 10% FBS Dulbecco's Modified Eagle Medium (Genesis Scietific, San Diego, CA) supplemented

with pTivator PSA, (Atlanta Biologicals, GA). The cells were grown and confluences were checked on regular basis. When confluence was close to 80%, cells were prepared for inoculation. Cells were counted using a hemocytometer and resuspended at a concentration of $3*10^6$ cells/ml in PBS. Mice were anaesthetized using isoflurane gas. While mice were unconscious, the flank region was shaved and $3*10^5$ PDAC4662 cells were injected subcutaneously in the flank region.

3.4 Chemotherapy and exercise interventions

All animals were acclimatized to the treadmill (Columbus Instruments) by walking at 8 meters/min for 5 min/day for 3 days prior to inoculation. After acclimatization period, mice were inoculated with PDAC4662 cells and remained in their home cage until the tumor volume reached ~$50mm^3$. Mice then began their assigned intervention. Mice assigned to treatment with gemcitabine received 15mg/kg intraperitoneally three days per week. Mice assigned to exercise began each session with treadmill speed at 8 meters/min. For Cont-LV and Cont-HV, the speed was gradually increased from 8 meters/min to 12 meters/min. For the HIIT, the treadmill speed was increased from 8meter/min to 20meters/min within 30seconds. Cont-LV exercise group mice ran on the treadmill for 15 min at 12 meters/min (60-70% VO_2max (Schefer and Talan, 1996), 5x weekly. Cont-HV exercise group mice ran on the treadmill for 45 min at 12 meters/min, 5x weekly. HIIT exercise group mice performed 10 intervals of 1 minute running at 20 meters/min (>90% VO_2max) followed by 1 minute walking at 8 meters/min, 3x weekly (Blackwell et al., 2018). Each exercise protocol is described in Table 3.2.

Table 3-2: Exercise Protocol

Groups	Intensity	Duration	Frequency
Continuous moderate intensity-Low volume (Cont-LV)	12meters/min	15 min	5 days/week
Continuous moderate intensity--High volume (Cont-HV)	12meters/min	45 min	5 days/week
High intensity Interval training (HIIT)	1 minute running at 20 meters/min followed by 1 minute walking at 8 meters/min	20 min	3 days/week

3.5 Assessment of tumor volume

Mice were examined three times each week (every 2 days) after tumor cell inoculation for the presence of tumors. Tumor size was measured using calipers. The tumor volume was calculated using this formula.

$$Tumor\ volume = (Length * width * width)\ /\ 2$$

3.6 Euthanasia procedure

On the euthanasia day, the mice were anaesthetized using isoflurane gas. When mice became unconsciousness, the blood was collected via cardiac puncture. A 25-gauge needle with 1ml syringe were inserted into the mouse's heart and slowly blood was taken from the heart to avoid collapsing of heart. Immediately after the blood collection mice

were killed via cervical dislocation. Once death was confirmed, spleen and tumor were isolated from the mouse within 20 minutes.

3.7 Immune measures

3.7.1 Isolation of lymphocytes from blood

Blood was collected via cardiac puncture and immediately transferred into the 1ml EDTA coated tubes. Aliquots of 50µl of whole blood were incubated with 2µl of CD3-PE-Vio770, CD4-Viogreen, and NKP46-FITC,(All: Milteny Biotech Inc. San Diego, CA) and 6µl of CD8a-APC Vio770 (Milteny Biotech Inc. San Diego, CA) for 20 min at room temperature. The blood was then incubated with 2ml of 1X RBC lysis buffer (Thermo fisher Scientific, Waltham, MA) for 15 min at room temperature. Following incubation, the blood was centrifuged at 400g for 10 min and washed twice-using PBS containing EDTA and resuspended into 200µl PBS prior to flow cytometry analysis (Milteny Biotech Inc. San Diego, CA).

3.7.2 Isolation of tumor-infiltrating lymphocytes

Tumors were isolated from mice after euthanasia. Immediately after isolation, tumors were cut into two equal sections. One part was utilized for vascular experiments (results are not included in this dissertation) and other part was transferred into ice-cold PBS and kept on ice until further analysis. Two different protocols were used to isolate the immune cells form tumors. For the first phase of experiment, a part of tumor was mechanically and chemically dissociated to produce single cell suspensions. To remove the blood clot, the tumor was washed 2-3 times with ice-cold PBS containing EDTA. After washing, the tumor was cut into multiple tiny small pieces using scissors. All the

pieces of the tumor were carefully transferred into a 50ml tube containing a freshly prepared collagenase mixture (40mg Worthington Type I Collagenase +0.3mg Worthington DNAase in 20ml HBBS solution (Thermo fisher Scientific, Waltham, MA)). The tumor with collagenase mixture was shaken at a speed of 300rpm at 37^0C for 30 min. After 30 minutes, 20ml RPMI media (Thermo fisher Scientific, Waltham, MA) supplemented with 10% FBS was added to stop the collagenase reaction. The tumor pieces were then passed through 100µm and 70µm strainers respectively (USA Scientific, Ocala, FL). The filtrate was centrifuged at 400g for 10 min. Red blood cells were lysed by incubating the cells in 3ml of 1X RBC lysis buffer (Thermo fisher Scientific, Waltham, MA) for 15 min at room temperature. Cells were washed twice with PBS containing EDTA and resuspended into 500µl PBS containing EDTA. Cells were incubated with 2µl of CD3-PE-Vio770, CD4-Viogreen, NKP46-FITC, (Milteny Biotech Inc. San Diego, CA) and 6µl of CD8a-APC Vio770 (Milteny Biotech Inc. San Diego, CA) for 20 minutes at room temperature and ran on the flow cytometer (Milteny Biotech Inc. San Diego, CA).

For the replication experiment (second phase of experiment), tumor was dissociated using a tumor dissociation kit (Milteny Biotech Inc. San Diego, CA). Tumors were cut into small pieces and transferred into gentle MACS tubes (Milteny Biotech Inc. San Diego, CA). Reagents were added according to manufacturer instructions and tubes containing tumor were kept at 37^0C for 35 min in the incubator. After 35 minutes, tubes were taken out and solution was passed through 70um strainer and filtrate was collected in 50 ml tube. The filtrate was then centrifuged at 300G for 7 minutes, washed and resuspended in PBS. Aliquot of 200µl were be incubated with 2µl of CD3-PE-Vio770,

CD4-Viogreen, NKP46-FITC, and and 6µl of CD8a-APC Vio770 for 20 min at room temperature and were then analyzed on the flow cytometer.

3.7.3 Isolation of lymphocytes from spleen

On the day of euthanasia, spleens were isolated from the mice and immediately placed into ice-cold PBS. The spleen was rinsed twice using ice-cold PBS containing EDTA to remove blood clots. The spleen was cut into 2-3 pieces and then passed through a 100µm and 70µm strainer and cell-containing filtrate collected in a 50ml tube. This filtrate was centrifuged at 400g for 10 min. The cells were incubated with 2ml of 1X RBC lysis buffer for 15 min at room temperature, centrifuged at 400g for 8 minutes, and washed twice with PBS containing EDTA and resuspended into 20ml PBS. Cells were then counted and cryopreserved for the CD107a degranulation assay.

3.7.4 Cryopreservation of Splenocytes

Splenocytes were washed twice to remove RBC lysis buffer and resuspended into 20ml PBS. Splenocytes were counted and resuspended in freezing medium (10% DMSO with FBS) at a concentration of $5x10^6$ to $1x10^7$ cells/ml. Cells were stored in a cell-freezing containing overnight at -80°C and then transferred into liquid nitrogen.

3.7.5 CD107a degranulation assay

Frozen splenocytes were thawed on the day of the CD107a degranulation assay. To thaw the cells, cryovials were taken out from the liquid nitrogen or from -80^0C and kept in water bath for less than a minute. As soon as ice melted, cells were transferred into a 50ml tube containing 10ml of RPMI with 10% FBS media. Cells were centrifuged at

400g for 10 minutes and resuspended in RPMI media at a final concentration of 3000cells/µl. Tumor target cells (PDAC-4662) were also washed and resuspended in RPMI media at a final concentration of 3000 cells/µl.

To perform the CD107a degranulation assay, 100µl of splenocytes ($3*10^5$ cells) were plated in a round-bottomed 96-well plate and rested for two hours at 37^0C. After two hours reagents were added: negative control (Splenocytes only), and experimental sample (Splenocytes and PDAC-4662 cells at 1:1 ratio). Subjects were tested in duplicate. 2ul CD107a-PE was added in each well. After one hour, monesin (Golgi Block, BD Bioscience) was added. Total volumes were equalized with RPMI media. Cells were incubated 6h at 37^0C in 5% CO_2. Cells were collected in a FACS tube, washed, and resuspended into 200ul PBS and stained with 2µl of CD3-PE-Vio770, CD4-Viogreen, CD8a-APC Vio770, and NKP46-FITC. Following a 20-minute incubation, cells were fixed and permeabilized using CytoPerm kit (BD Bioscience), and incubated 20 minutes with 2µl of IFNy-APC for intracellular IFN-y detection. Results were collected on the flow cytometer.

3.8 Statistical analysis

Prior to starting the experiments, a power analysis was performed using G power (Version 3.1.94, Franz Faul, Universitat Kiel, Germany) based on the previous research to determine the number of animals in each group (Schadler et al., 2016). In a previous study, there was a large effect of exercise on tumor growth relative to sedentary (Schadler et al., 2016). We expected similar differences between our exercise groups. Power analyses indicate that 6 animals per group would provide 80% power to detect differences

at $p<0.05$. All statistical analyses were performed using "Statistical Package for the Social Sciences" version 21 software (IBM Corp., Armonk, NY). Statistical significance was accepted at $p<0.05$. Normality was assessed by examining skewness and kurtosis.

First, we investigated the effects of gemcitabine treatment and exercise on body weight in chemotherapy experiment groups (Sed, Sed+Gem, Cont-LV+Gem, Cont-HV+Gem and HIIT+Gem). Body weight was assessed 7 times in replication 1 and 8 times in replication 2 during experiment. To examine the effect of intervention (exercise or treatment) on body weight over time, body weight of groups (Sed, Sed+Gem Cont-LV+Gem, Cont-HV+Gem and HIIT+Gem) was compared to each other using repeated measure ANOVA. *Bonferroni post hoc* test was used to compare the means of all possible pairs of the all measures. One-way ANOVA with *Bonferroni post hoc* correction was used to compare body weight between groups on each measurement day. Similarly, in experiment 'without chemotherapy', the body weights over time between groups (Sed, Cont-LV, Cont-HV and HIIT) was compared using repeated measure ANOVA. One-way ANOVA with *Bonferroni post hoc* correction was used to compare body weight between groups on each measurement day.

The first aim was to investigate the effects of three different exercise interventions on tumor growth during chemotherapy treatment. Tumor volume was assessed 7-8 times throughout the experiments. To examine the effect of gemcitabine on tumor volume, we compared Sed with Sed+Gem group. Change in tumor volume over time was assessed using repeated measure ANOVA. To compare tumor volume between groups on each measurement day, independent t-test was used. To examine the effect of exercise on tumor growth over time, tumor volume of each group (Cont-LV+Gem, Cont-HV+Gem,

HIIT+Gem, and Sed+Gem) was compared using repeated measures ANOVA. *Bonferroni post hoc* test was used to compare the means of possible pairs. To compare tumor volume between groups on each measurement day, one-way ANOVA with *Bonferroni post hoc* correction was used.

The second aim of this study was to investigate the effects of three different exercise interventions on number of lymphocytes (CD8+ T-cells, CD4+ T-cells, and NK cells) in blood and tumor and NK cell function in pancreatic-tumor bearing mice receiving chemotherapy. To compare the effect of gemcitabine on number of lymphocytes/NK cell function, Sed group was compared with Sed+Gem group using an independent *t*-test. To investigate the effect of exercise on the number of lymphocytes and on NK cell function, groups (Cont-LV+Gem, Cont-HV+Gem, HIIT+Gem, and Sed+Gem) were compared using one-way ANOVA with *Bonferroni post hoc* correction.

The third aim of this study was to investigate the effect of the three different exercise interventions on tumor growth without chemotherapy treatment. To examine the effect of exercise on tumor growth over time, tumor volume of each group (Cont-LV, Cont-HV, HIIT, and Sed) was compared using repeated measures ANOVA. *Bonferroni post hoc* test was used to compare the means of possible pairs. To compare tumor volume between groups on each measurement day, one-way ANOVA with *Bonferroni post hoc* correction was used.

The fourth aim of this study was to investigate the effects of the three different exercise interventions without gemcitabine treatment on the number of lymphocytes (CD8+T-cells, CD4+T-cells, and NK cells) in blood and tumor and NK cell function in pancreatic-tumor bearing mice. To investigate the effect of exercise on number of

lymphocytes/ NK cell function, groups (Cont-LV, Cont-HV, HIIT, and Sed) were compared to each other using one-way ANOVA with *Bonferroni post hoc* correction.

CHAPTER IV

4. RESULTS

4.1 Effects of exercise training interventions on tumor growth and immune system in gemcitabine-treated mice

4.1.1 Intervention assignment

Two independent replications were performed to investigate the effects of the three different exercise interventions on pancreatic tumor growth during gemcitabine treatment. For each replication, 32 six-week-old male C57BL/6J were inoculated with PDAC4662 cells. Tumors first became palpable (tumor volume ~ 55mm^3) approximately one week after inoculation (Replication 1: 8 days, n=30; Replication 2: 7 days, n=28). Mice with comparable tumor volume were then assigned to their treatment groups such that the average tumor volume within each group was equal (Table 4.1). Mice began their assigned intervention at this time (that is, 8 and 7 days after inoculation) and continued until they required euthanization due to tumor burden (~1200 mm^3) or other illnesses. In both replications, ~80% of mice required euthanization due to ulcer development, not because of tumor size. All interventions ended after two weeks. One mouse in HIIT+Gem in Replication 1 did not complete any exercise sessions and was excluded from analyses. In Replication 2, one mouse each in Sed+Gem, Cont-HV+Gem, and HIIT+Gem was removed from analyses because tumor volume did not increase after start of intervention. In addition, one mouse from Sed was euthanized on day 19 because of an ulcer.

Table 4-1: Tumor volume (mm^3) at start and end of intervention in gemcitabine-treated mice

Replication	Group	Start of Intervention		End of Intervention	
		N	Tumor volume (Mean ± SD)	N	Tumor volume (Mean ± SD)
1	Sed	6	58.2 ± 18.78	6	498.78 ± 271.21
	Sed+Gem	6	57.83 ± 15.57	6	268.63 ± 142.63
	Cont-LV+Gem	6	56.63 ± 10.91	6	226.98 ± 92.35
	Cont-HV+Gem	6	58.1 ± 27.39	6	328.40 ± 205.35
	HIIT+Gem	6	56.95 ± 27.06	5	211.68 ± 72.28
2	Sed	5	58.11 ± 19.65	4	479.68 ± 107.25
	Sed+Gem	5	56.72 ± 13.29	4	249.85 ± 123.7
	Cont-LV+Gem	5	52.92 ± 26.91	5	222.04 ± 71.31
	Cont-HV+Gem	5	55.6 ± 20.17	4	182.03 ± 78.55
	HIIT+Gem	5	52.19 ± 17.99	4	133.02 ± 36.15

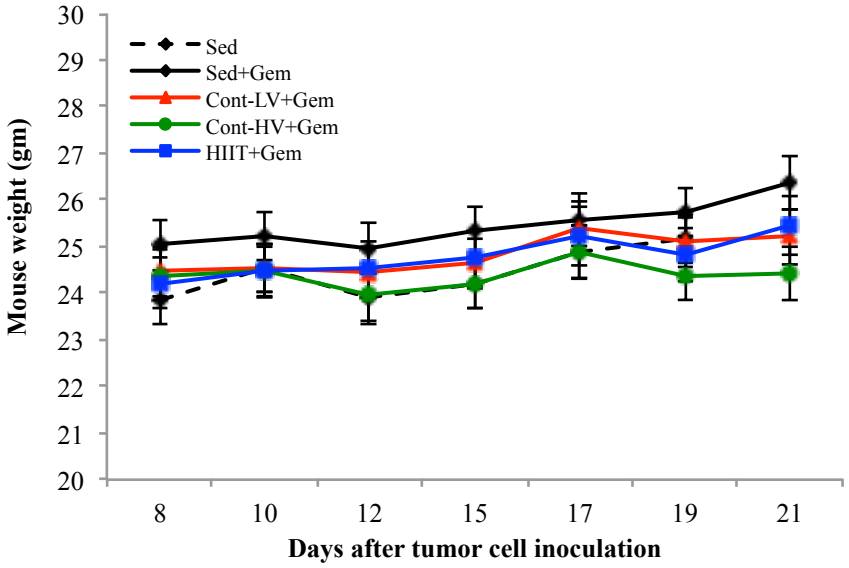
4.1.2 Effect of exercise training interventions on body weight in gemcitabine-treated mice

Mouse body weight over the course of the interventions is shown in Figure 4.1. To assess the effect of gemcitabine on body weight, Sed was compared with Sed+Gem. Weight significantly increased over time (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: Replication 1 ($F_{(2,20)} = 9.537$; $p = 0.001$, $\eta^2 = 0.488$); Replication 2 ($F_{(2.5,15)} = 14.733$; $p \leq 0.001$, $\eta^2 = 0.711$)). Weight did not significantly differ between the groups (Replication 1 = ($F_{(1, 10)} = 1.444$; $p = 0.257$, $\eta^2 = 0.126$); Replication 2 = ($F_{(1, 6)} =$

0.695; p = 0.436, $\eta^2 = 0.104$)). Independent t tests were used to compare weight between groups on each measurement day. There was no significant differences in weight between groups on final day (Replication 1 : ($t_{(10)} = -0.797$; p = 0.444); Replication 2 : ($t_{(6)} = 1.031$; p = 0.343)). On average, animals gained ~1.4 gm over the course of the two-week intervention.

To assess the effect of exercise training on body weight, each exercise group was compared to each other and to Sed+Gem. Again, weight significantly increased over time (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: Replication 1: ($F_{(2.6,50)} = 26.06$, $p \leq 0.001$, $\eta^2 = 0.578$; Replication 2: ($F_{(3,40)} = 13.427$, $p \leq 0.001$, $\eta^2 = 0.508$)). There was no significant difference between the groups overall (Replication 1: $F_{(3, 19)} = 0.778$, p = 0.521, $\eta^2 = 0.109$; Replication 2: ($F_{(3, 13)} = 0.564$; p = 0.649, $\eta^2 = 0.115$) or on the final measurement day (Replication 1: $F_{(3, 19)} = 1.885$, p = 0.166, $\eta^2 = 0.229$; Replication 2: $F_{(3, 13} = 0.781$; p = 0.525, $\eta^2 = 0.153$). On average, animals gained ~2gm over the course of the two-week intervention.

A.



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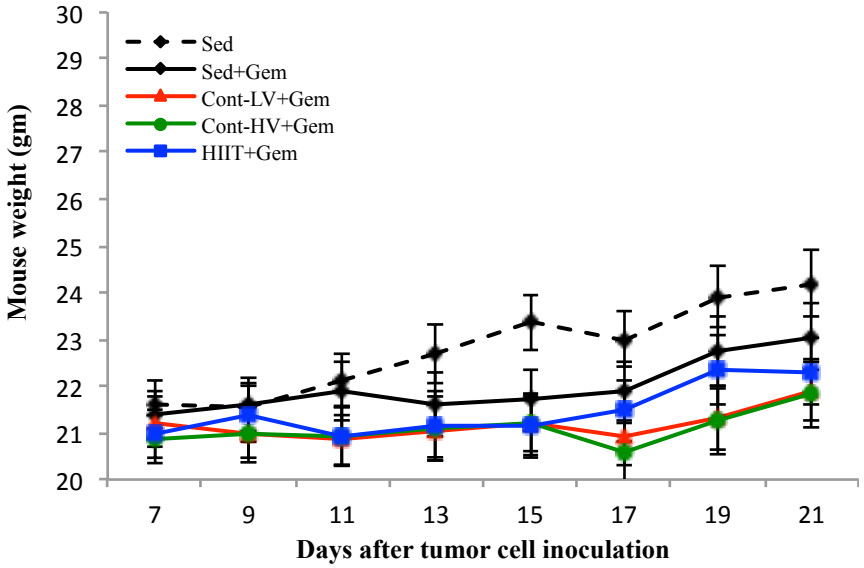


Figure 4.1 Changes in body weight of sedentary and exercised mice with and without gemcitabine treatment in (A) Replication1 (B) Replication 2. Mice weights were measured on the indicated days and are shown in mean ± SEM. N = 4 to 6 mice per group.

4.1.3 Effect of exercise training interventions on tumor growth in gemcitabine-treated mice

To assess the effect of gemcitabine on tumor growth, Sed was compared with Sed+Gem. Although tumor growth was not completely abrogated, gemcitabine treatment did slow growth (Figure 4.2). Tumor volume significantly increased over time (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: Replication 1: ($F_{(1,11)} = 18.64$; $p = 0.001$, $\eta^2 = 0.65$); Replication 2 ($F_{(2,13)} = 37.418$; $p \leq 0.001$, $\eta^2 = 0.86$). In Replication 1, gemcitabine significantly inhibited tumor growth overall (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: $F_{(1, 10)} = 8.279$; $p = 0.016$, $\eta^2 = 0.45$). Independent t tests were used to compare tumor volume between groups on each measurement day. There was a significant difference in tumor volume between groups first appear on day 15 (that is, 7 days after initiation of gemcitabine) ($t_{(10)} = 4.01$; $p=0.002$) and remain significant throughout the experiment (Day 19 $t_{(10)} = 2.787$; $p=0.019$) (Figure 4.2A). In replication 2, no overall significant reduction in tumor growth was observed with gemcitabine treatment ($F_{(1, 6)} = 3.419$; $p =0.114$, $\eta^2 = 0.363$). This may be due to low subject numbers in each group. On day 21, groups were significantly different ($t_{(6)} = 2.8$; $p=0.031$) (Figure 4.2B).

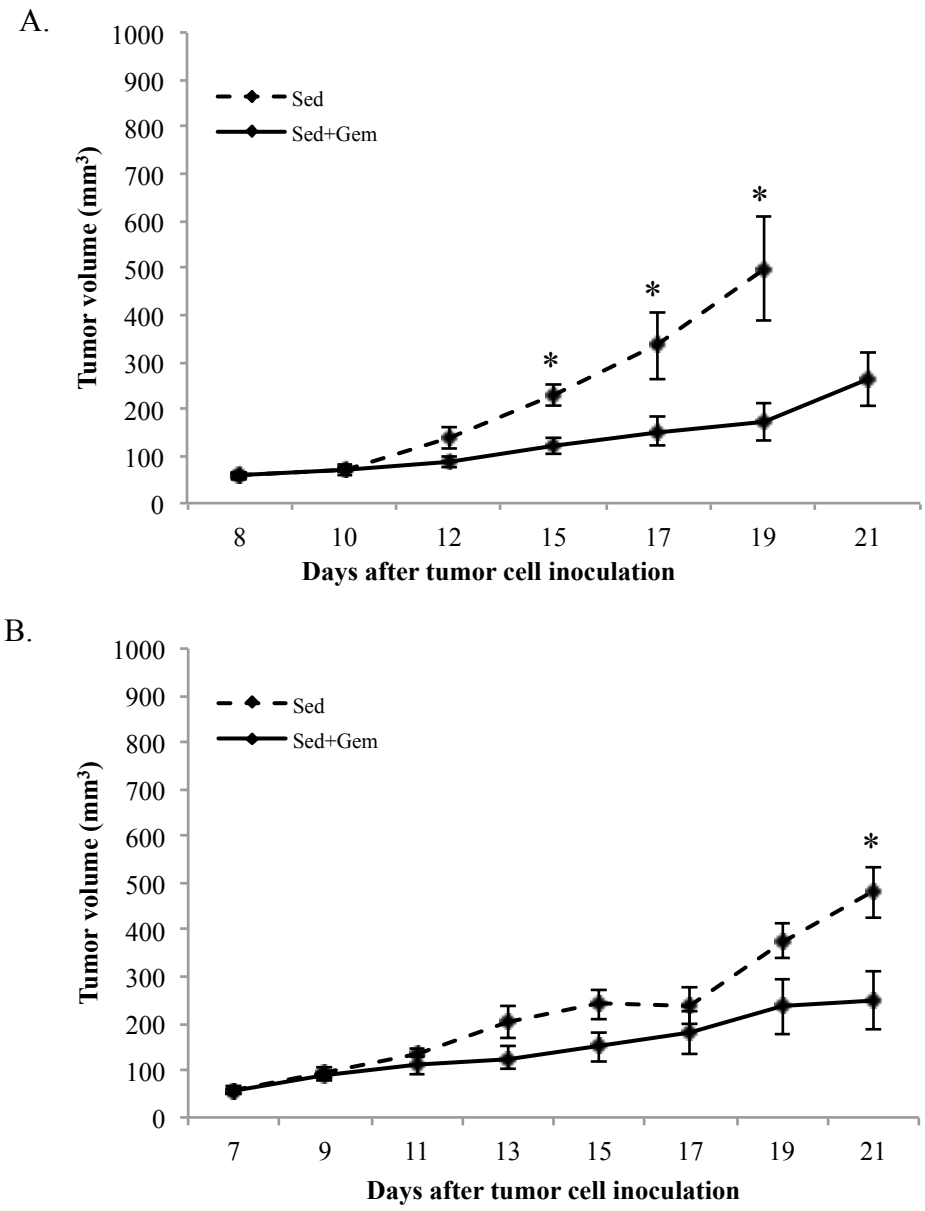


Figure 4.2. Tumor growth in sedentary mice with and without gemcitabine treatment in (A) Replication 1 (B) Replication 2. PDAC 4662 tumor volumes (mm^3) were measured on the indicated days and are shown in mean ± SEM. *$p<0.05$, N = 4 to 6 mice per group

To assess the effects of exercise training on tumor growth in gemcitabine-treated mice, each exercise group was compared to each other and to Sed+Gem (Figure 4.3).

Tumor volume significantly increased over time (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: Replication 1 ($F_{(1.5,28)} = 43.385$; $p \leq 0.001$, $\eta^2 = 0.695$); Replication 2 ($F_{(2,29)} = 27.80$; $p \leq 0.001$, $\eta^2 = 0.68$)). There were no significant differences between the groups (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: Replication 1: ($F_{(3, 19)} = 0.136$; $p = 0.937$), $\eta^2 = 0.021$); Replication 2: ($F_{(3, 13)} = 1.031$; $p = 0.411$, $\eta^2 = 0.192$). Comparing tumor volume using one-way ANOVA between groups on each measurement day also did not reveal significant group differences, even at the end of intervention (Replication 1: ($F_{(3, 19)} = 0.78$; $p = 0.520$, $\eta^2 = 0.11$); Replication 2: ($F_{(3, 13)} = 1.542$; $p = 0.251$, $\eta^2 = 0.262$).

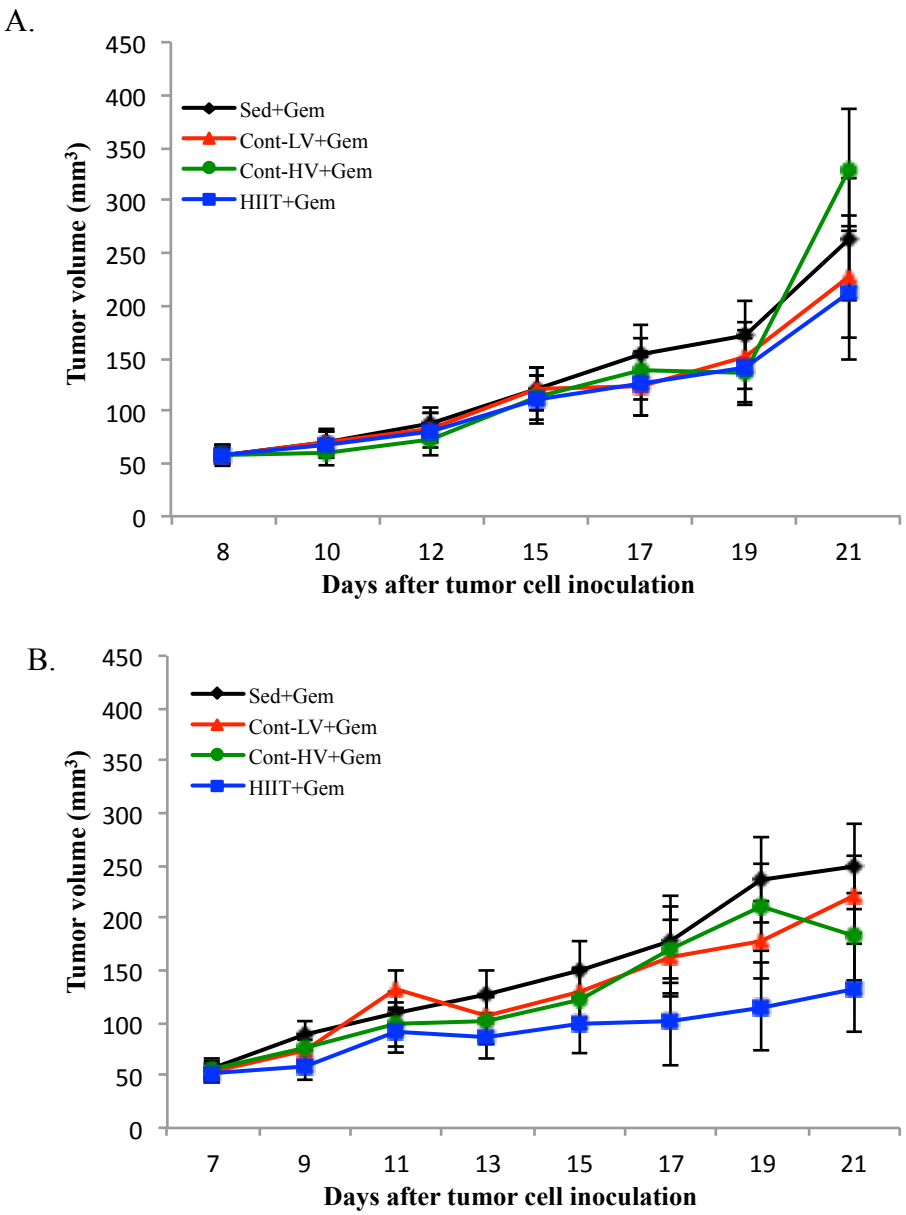


Figure 4.3. Tumor growth in sedentary and exercised mice with gemcitabine treatment in (A) Replication1 (B) Replication 2. PDAC 4662 tumor volumes (mm^3) were measured on the indicated days and are shown in mean ± SEM. N = 4 to 6 mice per group

To further compare the three exercise interventions to each other, we calculated the percentage change in tumor volume relative to Sed+Gem at the end of intervention (Figure 4.4). In Replication 1, Cont-HV+Gem appeared to be worse than Sed+Gem; this

relative increase in tumor volume was driven by a single outlier (compare Figure 4.4A to Figure 4.4B with outlier removed). In replication 1, HIIT+Gem was superior with a 19.39% reduction in tumor volume relative to Sed+Gem, followed by Cont-LV+Gem (-13.57%) and Cont-HV+Gem (-2.37%) (Figure 4.4B). In Replication 2, HIIT+Gem was again superior with a 46.76% reduction in tumor volume relative to Sed+Gem, followed by Cont-HV+Gem (-27.14%) and Cont-LV+Gem (-11.13%) (Figure 4.4C). We did not measure tumor weight in replication 1 and 2.

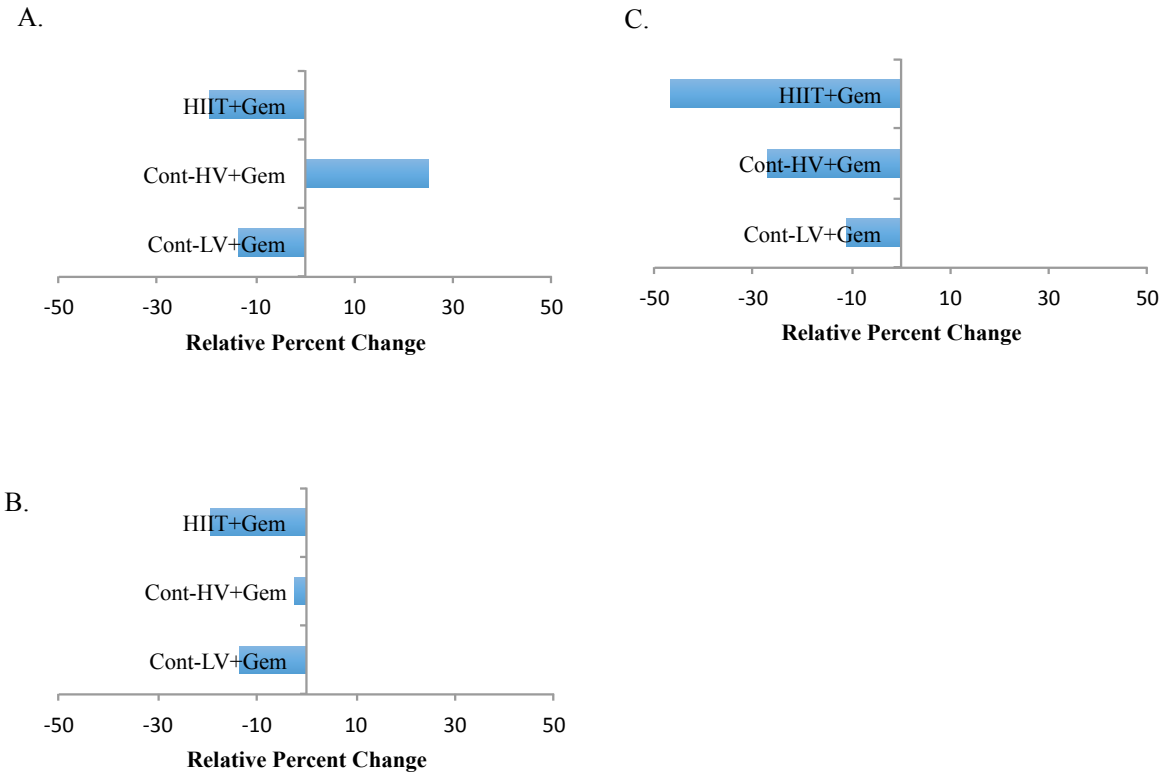
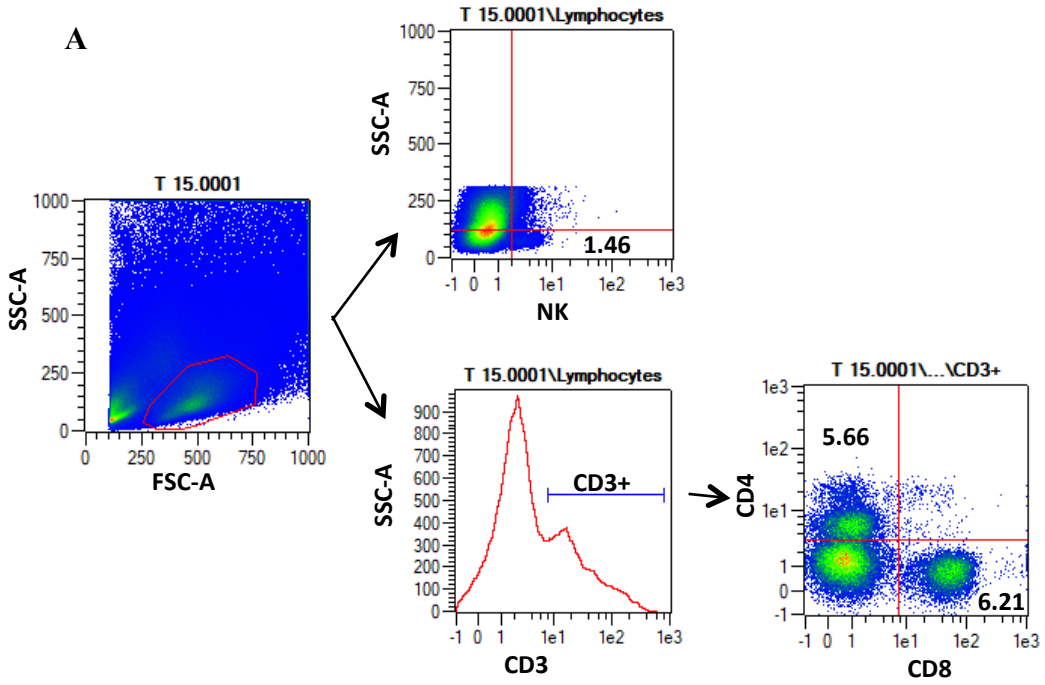


Figure 4.4. Relative percentage change in tumor growth compared to Sed+Gem. (A) Replication1 (B) Replication 1 with outlier removed (C) Replication 2. N = 4 to 6 mice per group

4.1.4 Effect of exercise training interventions on lymphocytes in gemcitabine-treated mice

We compared lymphocytes in tumor, blood, and spleen across sedentary and exercised animals. Using flow cytometry, we characterized lymphocytes found in tumor homogenates and in peripheral blood as NK-cells, CD4+ T-cells, or CD8+ T-cells. Representative flow cytometry plots are shown in Figure 4.5.



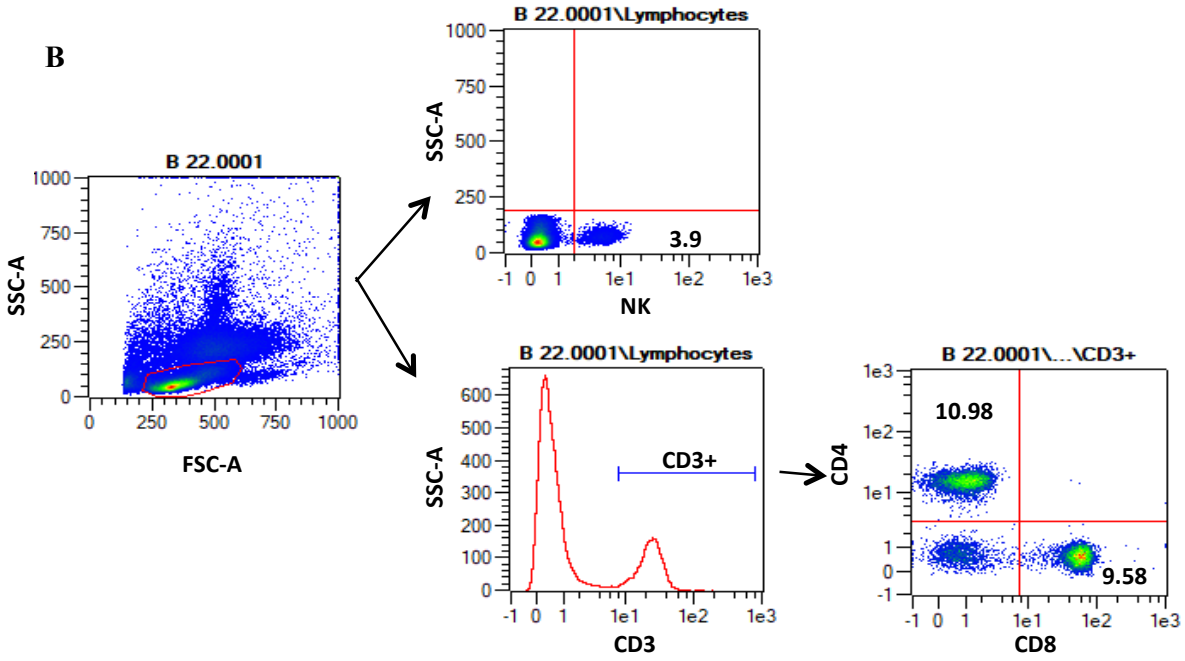


Figure 4.5. Representative flow cytometry dot plots in (A) Tumor and (B) Blood

First, we determined the effect of gemcitabine by comparing the proportions of tumor-infiltrating and blood lymphocyte subsets between Sed and Sed+Gem using independent *t* tests. The proportions of tumor infiltrating lymphocytes (NK-cells, CD4+T-cells, or CD8+ T-cells) did not significantly differ between groups (Figure 4.6A and C). Likewise, proportions of peripheral blood lymphocytes did not significantly differ between groups (Figure 4.6B and D).

We next assessed the effects of the exercise interventions on tumor infiltrating lymphocytes and peripheral blood lymphocytes. In replication 1, the proportions of tumor infiltrating lymphocytes did not significantly differ between groups (Figure 4.6A). Likewise, proportions of peripheral blood lymphocytes did not significantly differ between groups (Figure 4.6B). Replication 2 yielded similar results to replication 1. The

proportions of tumor infiltrating lymphocytes and peripheral blood lymphocytes did not significantly differ between groups (Figure 4.6C and D).

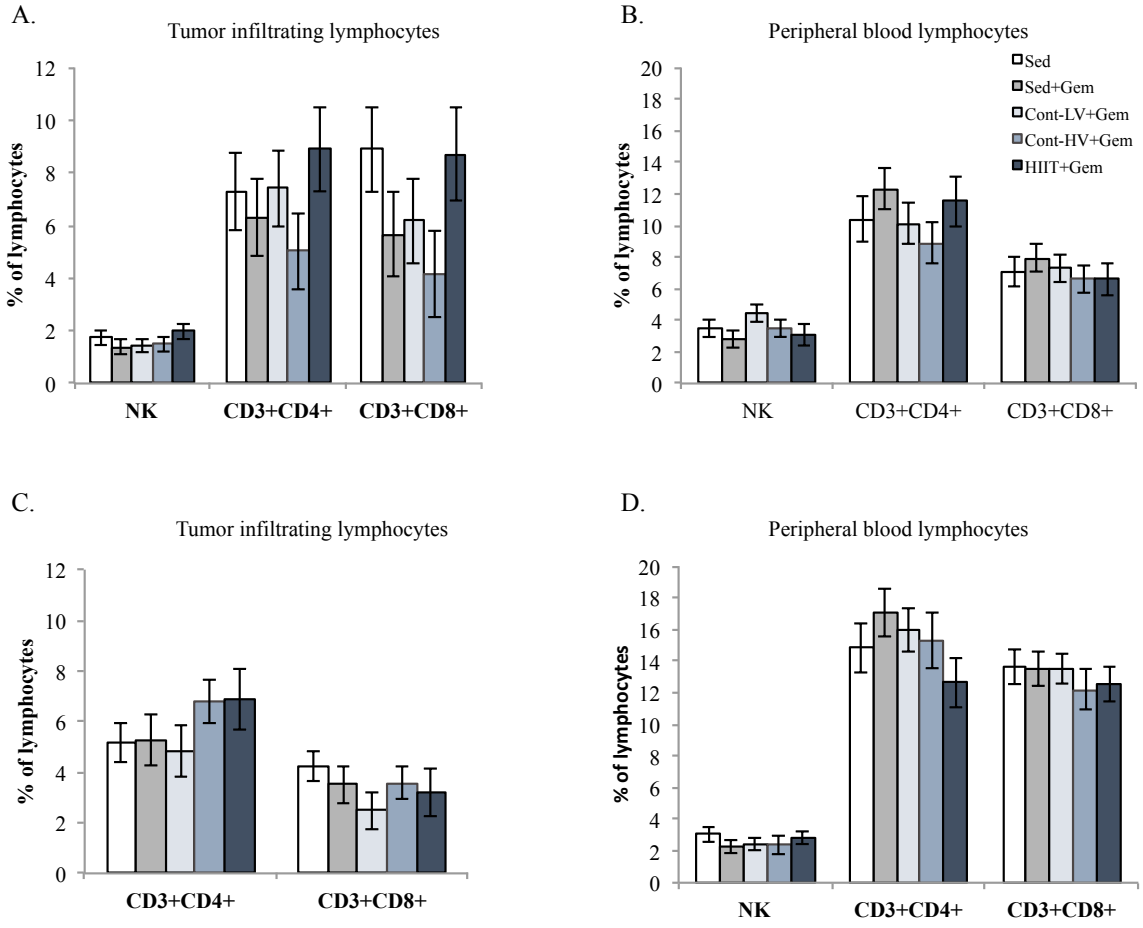


Figure 4.6. Tumor infiltrating lymphocytes in replication 1(A) and replication 2 (C) and peripheral blood lymphocytes in replication 1(B) and replication 2 (D) in sedentary and exercised mice with gemcitabine treatment. Data are shown in mean ± SEM.

4.1.5 Effect of exercise training interventions on NK cell function in gemcitabine-treated mice (CD107a degranulation assay)

We compared splenocyte NK cell function across sedentary and exercised animals. Flow cytometry was used to measure CD107a expression in NK cells after incubation

with PDAC4662 cells (experimental condition) or media (negative control). Representative flow cytometry plots are shown in Figure 4.7.

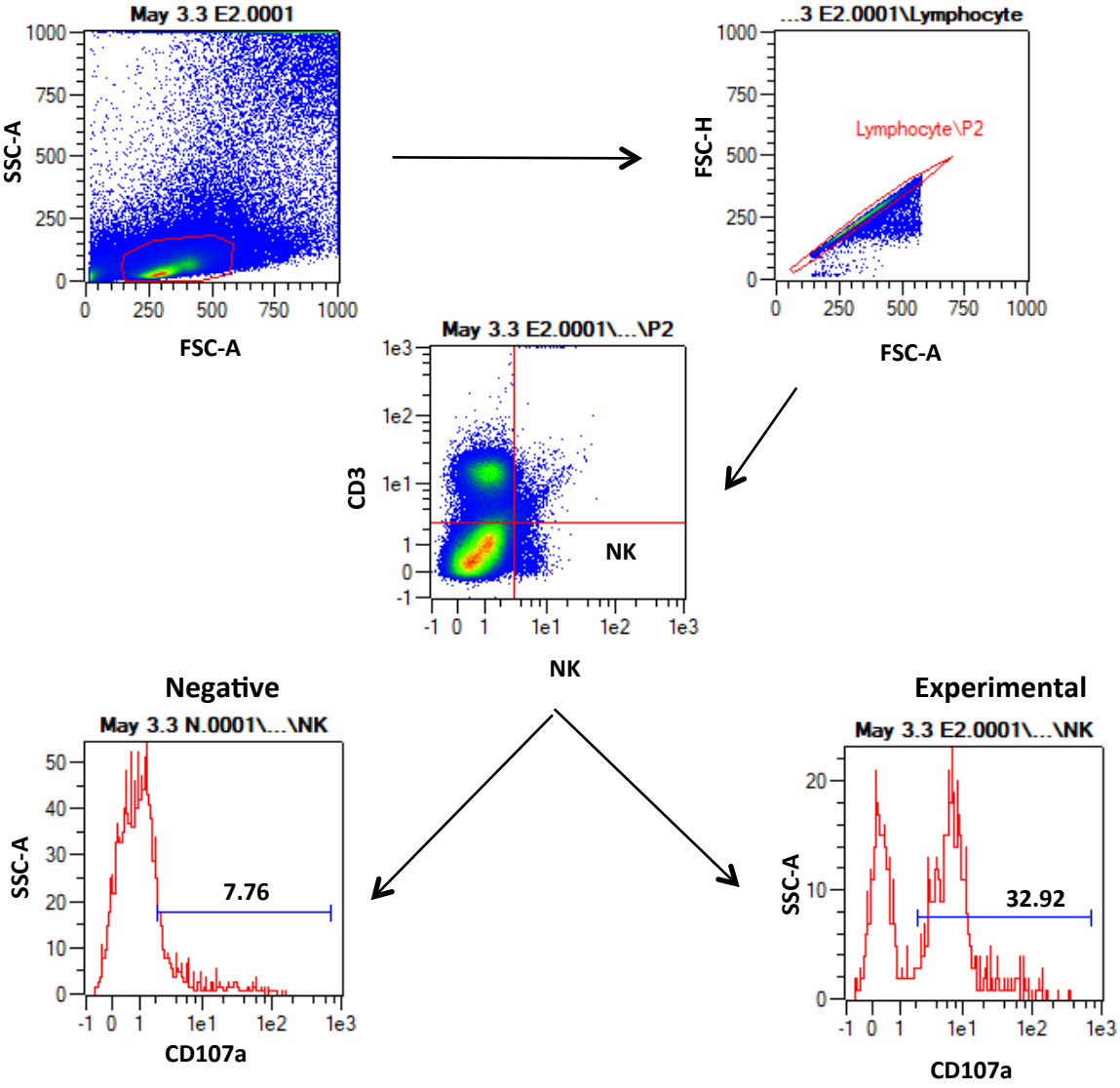


Figure 4.7. Representative flow cytometry plots of NK cell functional assay (CD107a degranulation assay)

In replication 1, 27 mice were included; one mouse from Sed and Cont-LV+Gem were not included. In replication 2, 21 mice were included; one mouse from Sed+Gem, Cont-LV+Gem, and Cont-HV+Gem group were not included due to insufficient numbers

of cells. The effect of gemcitabine on NK cell function was assessed by comparing CD107a expression between Sed and Sed+Gem. The CD107a proportion in NK cells did not differ between groups (Replication 1: $t_{(9)} = -0.1.393$; p=0.197; Replication 2: $t_{(7)} = -0.1.741$; p=0.125)(Figure 4.8A and B). We next compared CD107a expression between Sed+Gem, Cont-LV+Gem, CONT-HV+Gem, and HIIT+Gem. CD107a expression did not significantly differ between groups (Replication 1: $F_{(3, 18)} = 1.250$; p = 0.321, $\eta^2 = 0.172$; Replication 2: ($F_{(3, 12)} = 2.599$; p = 0.101, $\eta^2 = 0.394$) (Figure 4.8A and B).

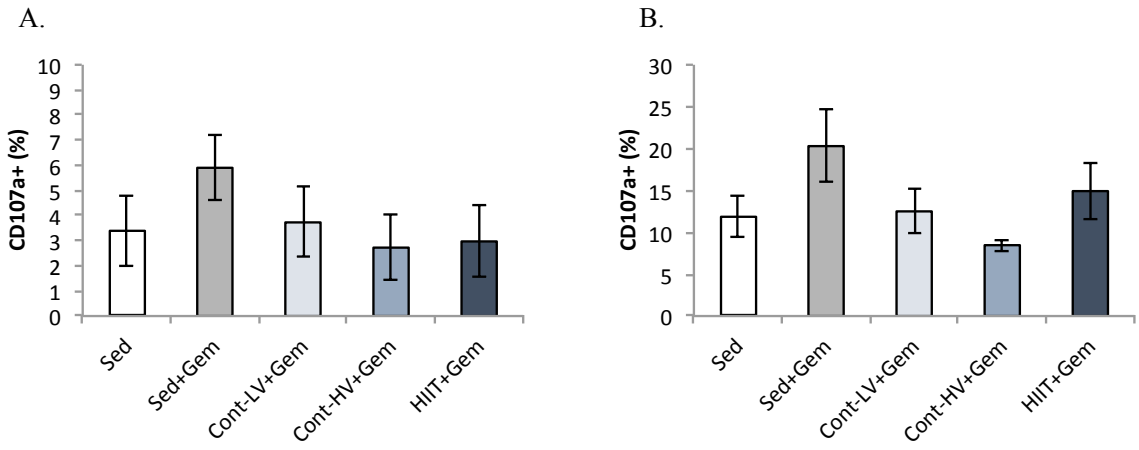


Figure 4.8. NK cell functional assay (CD107a degranulation assay) results in (A) replication 1 and (B) replication 2. Data are shown in mean ± SEM. * indicates significant difference from Sed, N = 4 to 6 mice per group

4.2 Effect of exercise training interventions on tumor growth and immune system in mice without gemcitabine treatment

The existing literature has more frequently focused on the effects of exercise on tumor growth in mice without the addition of chemotherapy. We therefore also examined the effects of the three exercise interventions on PDAC-4662 tumor growth in mice without chemotherapy treatment.

4.2.1 Intervention Assignment

Twenty-eight six-week-old male C57BL/6J were inoculated with PDAC-4662 cells. On day seven, tumor volume reached ~60mm^3 in most animals, and 24 mice were assigned to one of four groups such that the average tumor volume within each group was equal (N=6 per group) (Table 4.2). Three mice did not develop a tumor and one mouse developed a very large tumor (>150mm^3); these animals were excluded from the experiment. Mice began their assigned intervention on day eight and continued until they required euthanization due to tumor burden (1200 mm^3) or other illness. Sixty-seven percent (67%) of mice developed ulcers prior to tumor size reaching ~1200 mm^3. One mouse from HIIT and two mice from Cont-LV group were euthanized on days 16 and 18, respectively, due to ulcer development; all other mice completed their interventions on day 22. Two mice in Cont-HV and one mouse in HIIT refused to perform their assigned exercise and were excluded from all analyses.

Table 4-2: Tumor volume (mm^3) at start and end of intervention in mice without gemcitabine

Group	Mice assigned in each group		Start of Intervention		End of Intervention	
	N	Tumor volume (Mean ± SD)	N	Tumor volume (Mean ± SD)	N	Tumor volume (Mean ± SD)
Sed	6	65.15 ± 40.62	6	147.26 ± 73.05	6	844.76 ± 309.61
Cont-LV	6	65.6 ± 53.3	6	109.72 ± 36.24	4	422.37 ± 196.54
Cont-HV	6	66.3 ± 24.1	6	112.18 ± 27.29	4	726.69 ± 68.58
HIIT	6	65.7 ± 34	6	93.87 ± 33.12	4	918.12 ± 283.34

4.2.2 Effect of exercise training interventions on body weight in mice without gemcitabine treatment

Mouse body weight significantly increased over time (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: ($F_{(3,44)} = 44.288$; $p \leq 0.001$, $\eta^2 = 0.760$). There was no significant difference between the groups overall ($F_{(3, 14)} = 2.152$; $p = 0.139$, $\eta^2 = 0.316$) and on final measurement day ($F_{(3, 136} = 1.881$; $p = 0.179$, $\eta^2 = 0.287$). On average, animals gained 1.7 gm over the course of the two-week intervention (Figure 4.9).

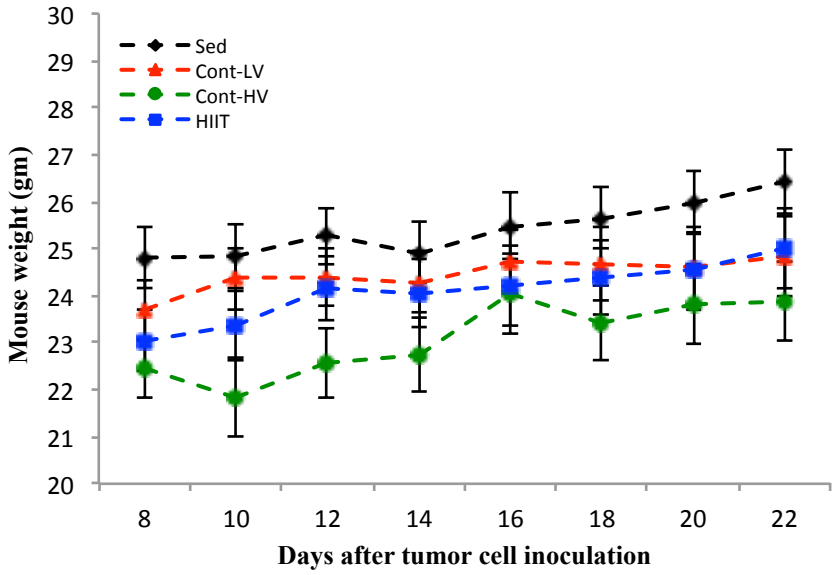


Figure 4.9. Changes in body weight of sedentary and exercised mice without gemcitabine treatment. Mice weights were measured on the indicated days and are shown in mean ± SEM. N = 4 to 6 mice per group

4.2.3 Effect of exercise training interventions on tumor growth in mice without gemcitabine treatment

Tumor volume significantly increased over time (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment ($F_{(3, 44)} = 44.876$; $p \leq 0.001$, $\eta^2 = 0.762$) (Figure 4.10A). A significant difference was observed between the groups (Repeated Measures ANOVA with *Greenhouse-Geisser* adjustment: ($F_{(3,14)} = 3.911$; $p = 0.032$, $\eta^2 = 0.456$). This overall group effect was likely due to Cont-LV, which exhibited slower tumor growth than Sed (p=0.059) and HIIT (p=0.055). Groups did not significantly differ on any measurement day, although there was a trend for a difference in tumor volume between groups on final measurement day ($F_{(3, 14)} = 3.28$; $p = 0.053$, $\eta^2 = 0.413$). Visual inspection of the data suggests this effect is driven by smaller tumors in Cont-LV group (Figure 4.10A).

To further compare the three exercise interventions to each other, we calculated the percentage change in tumor volume relative to Sed at the end of intervention (Figure 4.10B). Cont-LV was superior with a 50% reduction in tumor volume compared to Sed, followed by Cont-HV (-13.97%,). In contrast, HIIT had an increase in tumor volume relative to Sed (+8.68%) (Figure 4.10B). We also measured tumor weight at the end of experiment and did not find significant difference in tumor weight between groups ($F_{(3, 17)} = 1.024$; $p = 0.407$, $\eta^2 = 0.153$).

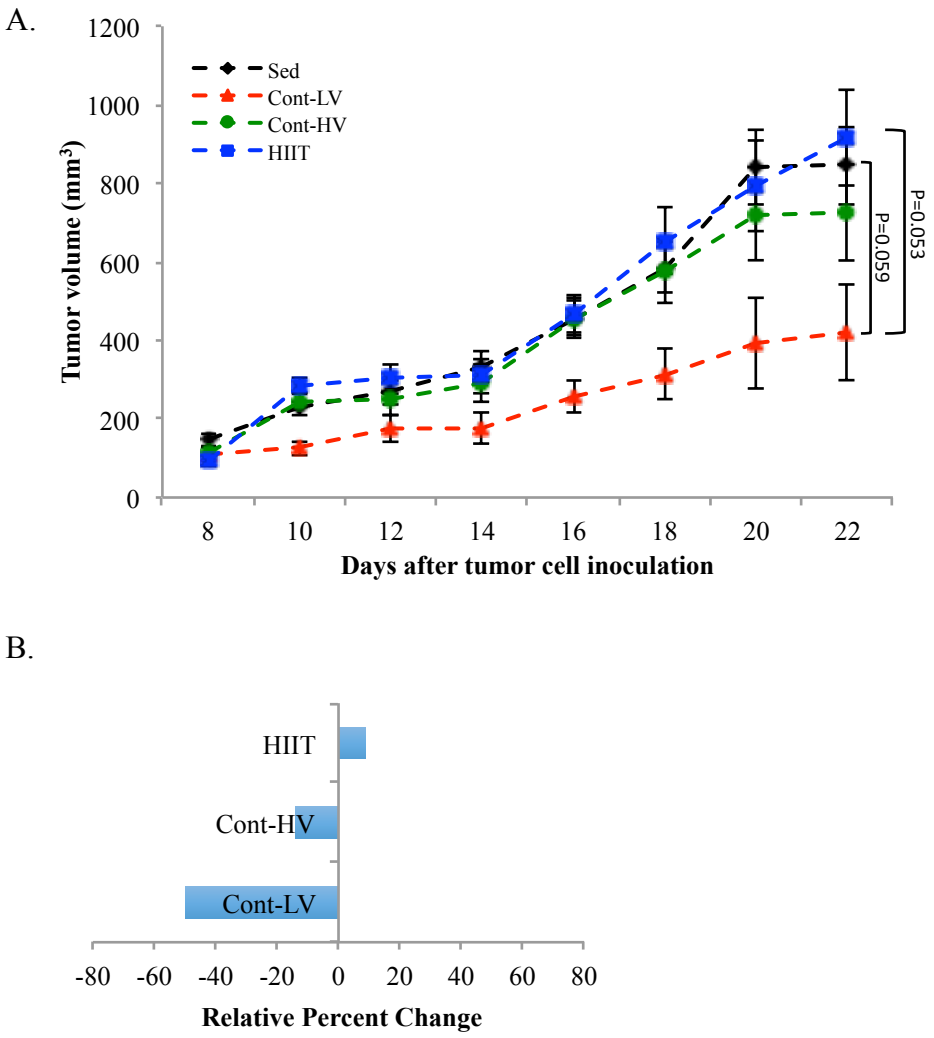


Figure 4.10. (A) Tumor growth and (B) Relative percentage change compared to sedentary mice without gemcitabine treatment. PDAC 4662 tumor volumes were measured on the indicated days and are shown in mean ± SEM. *p<0.05, N = 4 to 6 mice per group

4.2.4 Effect of exercise training interventions on lymphocytes in mice without gemcitabine treatment

As described earlier, 24 tumor-bearing mice were assigned to one of four groups, none receiving gemcitabine. Three mice, which refused to exercise, were excluded from

all analyses. One mouse from HIIT group only completed one week of exercise was also excluded from the analysis. In the remaining 20 mice, two mice from Cont-LV group were euthanized on day 18, due to ulcer development. All other mice were sacrificed on day 22.

We compared proportions of lymphocytes in tumor and blood between groups including all 20 animals. There was a significant difference in the proportion of tumor infiltrating CD8+ T-cells between groups ($F_{(3, 16)} = 4.986$; $p = 0.012$, $\eta^2 = 0.483$). Post-hoc analyses reveal that Cont-LV significantly differed from Sed (p=0.039) and Cont-HV group (p=0.039), but not from HIIT (p=0.067). There was no significant difference in tumor infiltrating NK and CD4+ T-cells (Figure 4.11A). Proportions of peripheral blood lymphocytes did not significantly differ between groups (Figure 4.11B).

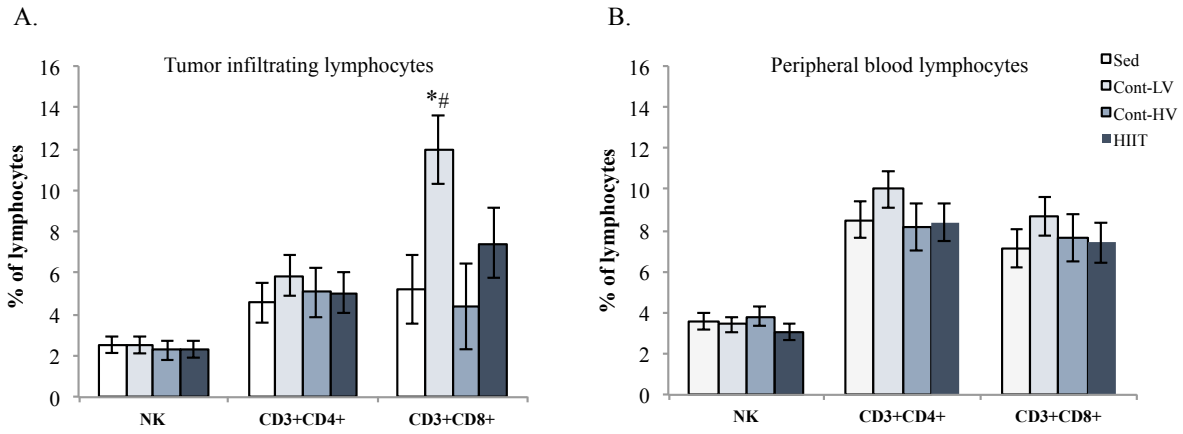


Figure 4.11. Tumor infiltrating lymphocytes (A) and peripheral blood lymphocytes (B) in sedentary and exercised mice without gemcitabine treatment. Data are shown in mean ± SEM. * indicates significant difference from Sed, # indicates significant difference from Cont-HV. N = 4 to 6 mice per group

To further understand the relationships between tumor-infiltrating lymphocytes and tumor growth in our pancreatic model, we performed a Pearson Bivariate correlation test including all 21 animals. Tumor volume was inversely correlated with CD8+ T-cell ($r = -0.601$, $p<0.01$) (Table 4.3).

Table 4-3: Correlation matrix indicating relationship between tumor volume and tumor infiltrating lymphocytes. Data is represented as Pearson correlation coefficient (p-value), * indicates significant correlation.

	Tumor volume (mm^3)	CD8+ T-cells	CD4+ T-cells	NK
Tumor volume (mm^3)	1			
CD8+ T-cells	-0.601 (p=0.005)*	1		
CD4+ T-cells	-0.342 (p=0.140)	0.695 (p<0.01)*	1	
NK	-0.225 (p=0.339)	0.578 (p=0.008)*	0.804 (p<0.01)*	1

4.2.5 Effect of exercise training interventions on NK cell function in mice without gemcitabine treatment (CD107a degranulation assay)

Splenocytes from 18 mice were included in these CD107a degranulation assays; one mouse from each Sed and Cont-HV were not included due to insufficient numbers of cells. CD107a expression did not significantly differ between groups ($F_{(3, 15)} = 1.193$; $p = 0.346$, $\eta^2 = 0.193$) (Figure 4.12).

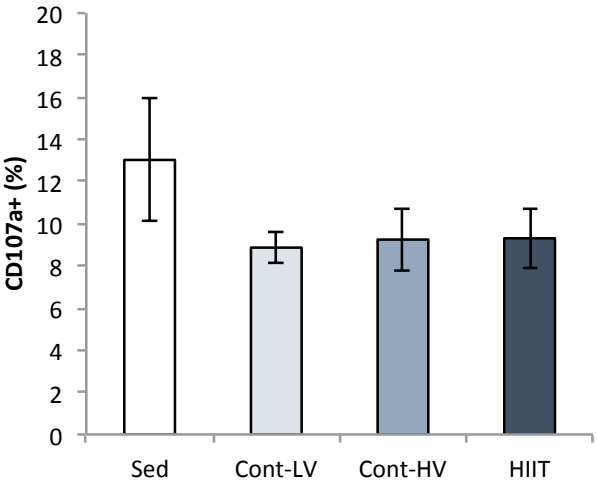


Figure 4.12. NK cell functional assay (CD107a degranulation assay). Data are shown in mean ± SEM. N = 3 to 6 mice per group

CHAPTER V

5. DISCUSSION

In this study, we examined the effects of the three different exercise interventions on tumor growth in a mouse model of pancreatic cancer. We report no difference in tumor volume between the exercise groups and sedentary mice in the experiments that also provided gemcitabine treatment. In contrast, we did find a significant effect of exercise in tumor-bearing mice not treated with gemcitabine, potentially driven by the tumor-inhibiting effect of continuous low-volume exercise. These effects were accompanied by an increase in the CD8+ T-cells in tumors in the continuous low volume exercise group. These results support that exercise can suppress the pancreatic tumor growth by promoting CD8 T-cell infiltration in tumor.

5.1 Effect of exercise training interventions on pancreatic tumor growth and immune system in gemcitabine treated mice

5.1.1 Exercise effects on pancreatic tumor growth in gemcitabine-treated mice

In the existing literature, most of the studies investigated the effect of exercise on tumor growth without providing any additional treatment (such as chemotherapy). In one study, the effects of different exercise intensities in a rat breast cancer model were compared. There was a decrease in the number of tumors with an increase in training intensity (Malicka et al., 2015). This supports that different exercise training interventions may yield different results on tumor growth. In clinical settings, the majority of cancer patients receive one or more cancer treatments (e.g., surgery, chemotherapy, radiotherapy,

immunotherapy, and/or anti-hormonal therapy), and therefore it is imperative to understand the effect of exercise alongside conventional and novel cancer therapies. Thus, we compared three exercise interventions provided at the same time as chemotherapy treatment. In the present study, we hypothesized that exercise of greater intensity and longer duration would yield slower tumor growth with chemotherapy, such that tumor growth would be slowest in HIIT+Gem, followed by Cont-HV+Gem, and then Cont-LV+Gem. However, this hypothesis was not supported, as we did not report any difference between groups.

Our initial hypothesis was rooted in the existing literature. Previously, Betof and colleagues reported that voluntary exercise inhibited tumor growth in mice, and that this likely occurred as exercise normalized the vascular system by favorably modulating the three primary determinants of intra-tumoral hypoxia: tumor cell mass, apoptosis, and vessel density. In these experiments, exercise reduced 4T1 mammary tumor growth and this reduction was associated with increased tumor cell apoptosis, increased microvessel density, vessel maturity, and perfusion, and reduced hypoxia (Betof et al., 2015). Further, voluntary exercise with chemotherapy suppressed tumor growth more effectively compared to exercise or chemotherapy alone. Later, Schadler and colleagues have shown that exercise-induced shear stress activates calcineurin-NFAT-TSP1 signaling in endothelial cells, which activates downstream anti-angiogenic protein thrombospondin-1 (TSP-1) and promotes vascular normalization in mice. Similar to the previous study by Betof et al, they also reported that exercise with chemotherapy caused a significantly greater decrease in PDAC 4662 pancreatic and B16 melanoma tumor growth than chemotherapy alone (Schadler et al., 2016). Together, these studies support that exercise

can normalize the vasculature, resulting in better drug delivery and thus inhibit tumor growth. Recently, Weenerberg and colleagues reported that 30-minutse of exercise at 18 m/min for 5days/week with PD-1 blockade and focal radiotherapy significantly reduced tumor growth in mice with established 4T1 mammary carcinoma. The reduction was greater with exercise compared to PD-1 blockade and focal radiotherapy therapy alone. Exercise training following RT/aPD-1 therapy reduced intratumoral myeloid-derived suppressor cells (MDSCs) accumulation and NK cell activation resulting in smaller tumor volume (Wennerberg et al., 2020)**.** Unlike these studies, we did not find any significant effect of exercise on pancreatic tumor growth in mice. We expect that this discrepancy between our results and those reported previously may be explained by differences that accumulated within the PDAC4662 tumor cell line with time, the timing of the intervention relative to tumor growth (exercise initiated before, immediately after, and initiated when tumor established), the duration and volume of the exercise intervention, and the type of exercise (voluntary vs. forced). Each of these potential explanations is discussed further below.

The current study was modeled in part after the experiments of Schadler et al. (Schadler et al., 2016). Specifically, we used the same cell line (PDAC 4662), dose of gemcitabine, and our Cont-HV replicated the exercise intervention (45 minutes for 5 days/week). To our surprise, we did not find differences in tumor growth in Cont-HV exercise training compared to sedentary groups in gemcitabine-treated mice. One potential explanation for this discrepancy might be changes in the PDAC-4662 tumor cells that were used in the earlier experiments described in Schadler et al. 2016 and those in the current study. It has been reported that continuous proliferation of cells may change the

phenotype and genotype of cell lines and reduce tumorigenicity of cell lines (Maitra et al., 2005; Neumann et al., 2010). It is possible that many cell passages accumulated between the initiation of the current experiments and those of Schadler et al. 2016. There is a possibility that the tumor cells used in replication 1 and 2 may have become less tumorigenic and more sensitive to gemcitabine. In support of this hypothesis, the tumors in the present study grew relatively slower (in our sedentary groups and sedentary with chemotherapy) than in the previously reported study (Schadler et al., 2016). There is a possibility that any positive effect of exercise training in our experiment was masked by the strong gemcitabine response. Another possible explanation for the discrepancy between our results and those reported previously may arise due to change in animals's microbiota. Animals lives in separate facilities have different husbandry, barrier conditions and dietary intake, which is known to change the animal's microbiome (Choo et al., 2017). These microbes can influence tumor progression via chronic activation of inflammation (Francescone et al., 2014). Therefore it is a possibility that mice used in current study may respond differently compared to previous study. Thus, we were not able to identify the differences.

Another possible explanation for the discrepancy between our results and those reported previously may arise from a difference in the timing of the intervention relative to tumor growth. Schadler and colleagues began exercising mice when tumor volumes reached ~35mm^3, whereas, in the present study, mice started exercising when tumor volumes reached ~55mm^3. It is possible that exercise has more pronounced beneficial effects when initiated at the early stage of the disease, and these beneficial effects are mitigated as the disease progresses. In our knowledge, no study has directly compared the

effects of the intervention timing relative to tumor growth. However, a few studies have compared the effects of exercise training begun before and after tumor inoculation without chemotherapy on tumor growth. Pederson and colleagues report a delay in the progression of fast-growing B16 melanoma tumors in mice when exercise was initiated prior to tumor cell inoculation, but no effect on tumor growth when exercise was initiated after tumor cell inoculation. In contrast, initiating running after tumor cell inoculation was sufficient to control tumor progression in slow-growing DEN-induced and Tg(Grm1) Epv models (Pedersen et al., 2016). The authors conclude that, in fast-growing tumor models, exercise initiated prior to tumor cell inoculation acted to prime the immune system to kill cancer cells at the inoculation site (Pedersen et al., 2016). Shalamzari and colleagues reported reduced mammary tumor volume in mice that started treadmill running prior to tumor cell inoculation and continued till euthanization, compared to mice that started exercise after tumor cell inoculation (Shalamzari et al., 2014). Another study that investigated the effect of exercise timing in mice reported that voluntary exercise prior to or during azoxymethane (AOM; a colon cancer inducing carcinogen) treatment resulted in a significant reduction in tumor number, but exercise following AOM exposure had no effect (Kelly et al., 2017). PDAC is a fast-growing tumor type, and there is a possibility that the delay in the timing of exercise initiation might change tumor outcomes. More research will be needed to understand the effect of timing of exercise initiation relative to tumor growth.

Previously, Wennerberg and colleagues demonstrated that three-weeks of treadmill running (30 minute for 5 days/week at 18 m/min) initiated when tumors first became palpable (8 days after 4T1 mammary tumor cell inoculation) with PD-1 blockade and

focal radiotherapy inhibited tumor growth more effectively compared to sedentary animals (Wennerberg et al., 2020). One group in our study (HIIT) ran on the treadmill at for 20 minutes at intervals of 20m/min, 3 days/week for two weeks, which is exercise intensity comparable to the Wennerberg's study. However, we did not find any significant difference between HIIT and sedentary animals. We cannot deny the fact that both studies investigated different tumors and used different treatment options that may affect tumor outcomes. However, there is also a possibility that the differences in exercise volume and total intervention duration played a role in the differing results. In particular, in the present study, animals in HIIT accumulated only six sessions of exercise prior to euthanization. Two-weeks of exercise training might not have been long enough to reduce tumor volume effectively. In future, we suggest using longer exercise intervention.

Another important consideration is difference in type of exercise. Previously, Betof and colleagues studies have examined the effect of voluntary exercise and reported reduced tumor growth (Betof et al., 2015). In contrast, we used forced treadmill so as to control the exercise duration and intensity. However, forced treadmill running is a stressful activity for the mice. It is possible that the stress generated by forced treadmill running transiently increased catecholamines and glucocorticoids such as cortisol in an intensity-dependent manner (Deusterab et al., 1989). Simone and colleagues have shown that glucocorticoids, even at physiological concentrations, enhance tumor cell proliferation by nearly twofold (Simon et al., 1984). Furthermore, cortisol may also act synergistically with catecholamine to facilitate tumor growth (Moreno-Smith et al., 2010). Thus, it is plausible that stressful situations characterized by both elevated catecholamine and cortisol levels may have promoted tumor growth. We did not measure catecholamines

or glucocorticoids in the current study, which may be a useful consideration for future studies. It should be noted however, that none of the exercise interventions used here led to significantly larger tumors.

5.1.2 Exercise effects on immune system in gemcitabine treated mice

NK cells represent a critical component of the innate immune defense, recognizing transformed cells independently of antibodies or major histocompatibility complex (MHC) restriction (Brodin et al., 2012; Vivier et al., 2012), while T-cells are cytotoxic effector cells of the adaptive immune response. It is well known that exercise mobilizes lymphocytes into peripheral blood and that this mobilization is proportional to exercise intensity and duration (Shephard and Shek, 1999; Walsh et al., 2011). NK cells are the most responsive cells to the exercise-dependent mobilization, followed by CD8+ T-cells, CD4+ T-cells, and lastly B cells, which respond poorly to exercise (Walsh et al., 2011). Natural Killer cell activity (NKCA) is enhanced by exercise in an intensity-dependent manner. Nieman and colleagues have shown that high intensity exercise yields higher NKCA compared to moderate intensity in young healthy males (Nieman et al., 1993b), and we have shown increased NKCA at an individual cell level during recovery from an exercise bout performed by healthy adults (Gupta et al., 2018). Exercise training may also yield enduring responses in NK cells. For example, in mice, exercise training increases NK cell populations in a variety of tissues (Pedersen et al., 2016). We hypothesized that exercise would mobilize lymphocytes into peripheral blood and thus increase the proportion of lymphocytes infiltrating into the tumors, with greater effects present with higher intensity (HIIT) and/or longer duration (Cont-HV). We further hypothesized that

the regular bouts of increased NKCA accompanying exercise would improve NK killing capacity of the tumor cells. However, we did not find any change in peripheral blood and tumor infiltrating lymphocytes. We also did not detect differences in NK cell activity between groups.

To our knowledge, only one study has measured immune responses to an exercise intervention with concomitant chemotherapy in pancreatic-tumor bearing mice and reported no change in CD8+ T-cells (Schadler et al., 2016). Consistent with this study, we also did not find any change in lymphocyte proportions in tumors from gemcitabine-treated mice. In contrast, Wennerberg and colleagues did report an increase in splenic CD8+ T-cells and NK cells as well as tumor-infiltrating CD69+ NK cells (activated NK cells) in their mouse model of breast cancer following three weeks of exercise training along with PD-1 blockade and focal radiotherapy (Wennerberg et al., 2020). They further reported that exercise reduced the tumor-induced accumulation of myeloid-derived suppressor cells (MDSCs) and this reduction in MDSCs was accompanied by a relative increase in NK and CD8+ T-cell activation, suggesting that exercise restores a favorable immune environment (Wennerberg et al., 2020). Just as the differences in exercise volume and intervention duration between our study and that of Wennerberg et al. could explain differences in the effect of exercise on tumor growth, so too could these explain the differences in the immune results. In particular, as we did not observe differences between the groups in our study (which differed in volume), it seems that two-weeks of exercise training may not be able to improve immune responses against tumors. We recommend using longer exercise interventions for future studies.

As discussed earlier, exercise training may have a more pronounced effect when started prior to tumor cell inoculation. To our knowledge, no study has directly assessed the effect of exercise timing relative to tumor growth with chemotherapy treatment on immune response. However, a study in which mice initiated exercise prior to tumor cell inoculation reported increased infiltration of NK and CD3 T-cells in tumors (Pedersen et al., 2016). There is a possibility that exercise initiated early primed the immune system for the tumor challenge, and potentially increased their killing of cancer cells at the inoculation site prior to amassing into a melanoma tumor (Pedersen et al., 2016). It is also possible that primed cytotoxic NK and CD8 + T-cells mobilized by exercise recognized and eliminated the more immunogenic cancer cells more effectively at early stage of tumor development (Gonzalez et al., 2018). As the current study began exercise after tumors were palpable (tumor volume = ~55mm^3), the tumor cells may already have generated an immunosuppressive microenvironment within the tumor, making it harder for infiltrating lymphocytes to survive and proliferate. We unfortunately did not measure immunoregulatory cells within the tumors, such as MDSCs, Tumor Associated Macrophages, or regulatory T-cells. It would be interesting for future studies to examine if exercise training begun before tumor cell inoculation or just prior to tumor detection improves immune surveillance and cytotoxicity against PDAC4662 cells.

To our knowledge, no study as yet investigated the effect of voluntary vs. forced treadmill exercise on the immune system with chemotherapy treatment. It is known that forced treadmill exercise generates a stress response in mice resulting increased stress hormone production (Griesbach et al., 2012). While acute stress increases T-cell mobilization through a β2-adrenergically mediated process, this process is blunted during

chronic stress (Dragoş and Tănăsescu, 2010), meaning that if the mice in the current study experienced the forced exercise as a chronic stress, the ability of their T-cells to mobilize to the tumor may have been reduced. This could explain why we did not observe a greater number of T-cells in the tumors of the exercised mice. Cortisol, another hormone increased by chronic stress, is also known to reduce NKCA via suppressing perforin and granzyme b production (Eddy et al., 2014).Thus, if the exercise intervention acted as a chronic stressor, we would expected decreased NKCA in these mice. However, while we did not see increased NK activity (indicated as degranulation response to tumor cells) in exercised mice, we did not see a decrease either. Future studies examining immune activity in mice with a forced exercise protocol are recommended to measure the stress hormone response to help understand underlying mechanisms.

The methods used to investigate immune responses may have a large impact on the results. In a review, Zimmer and colleagues have reported that the different methodological approaches used to assess NK-cell cytotoxicity produce different results (Zimmer et al., 2017). As one example, NK cell function may be measured by simply calculating the number of dead target cells following 4h incubation with NK cells. In this study, we instead measured NK cell function by measuring CD107a degranulation after incubating the NK cells with the tumor target cells. NK cell degranulation is a prerequisite for NK cell cytotoxicity, however assessment of degranulation does not necessarily correlate with target cell lysis. Target cell lysis depends not only on the extent of effector cell degranulation, but also on the content of secretory lysosomes, target cell structures that facilitate adhesion, and polarized secretion of secretory granules, in addition to the target cell's intrinsic sensitivity to NK cell-mediated death pathways (Bryceson et al.,

2005). Another important methodological consideration is how tumor tissue is processed during the extraction of immune cells, as well as the starting size of the tumor. In the current study, we used two different methods to isolate tumor-infiltrating lymphocytes across replication 1 and replication 2, where the method in replication 2 was a longer process. We expect that the delay in processing and analysis might have caused the death of tumor-infiltrating lymphocytes, as we noticed a rate of death of tumor-infiltrating lymphocytes in replication 2.

In our study, we were not able to detect IFN-γ secretion by NK cells. Our assays were based on previous reports that incubating NK cells with tumor cell in the presence of IL-2 for 6 hours yields detectable levels of IFN-γ (Freund-Brown et al., 2017; Freund et al., 2016). While our laboratory successfully measures intracellular cytokine expression in other experiments, we were unable to detect IFN-γ in this project. One difference in the current study and the others is that here we used frozen splenocytes, rather than fresh samples. There is a possibility that frozen cells do not respond to stimuli in the same manner as fresh cells. Previously it has been demonstrated that cryopreserved NK cells are less cytotoxic compare to fresh NK cells (Miller et al., 2014) and exhibited poor potency and recovery post-thaw (Szmania et al., 2015). In future experiments, we recommend using freshly obtained splenocytes for NK cell functional assays.

5.1.3 Gemcitabine effects on tumor growth and immune system in gemcitabine-treated mice

Gemcitabine significantly reduced tumor volume in our experiments, as demonstrated by our comparisons between Sed and Sed+Gem. Gemcitabine is a first-line

anti-cancer chemotherapy drug. The anti-tumor effect of gemcitabine has been observed across several different tumor types, including breast, lung, melanoma, and pancreatic tumors in mice (Schadler et al., 2016; Wang et al., 2004). This effect may be due mostly to a direct cytotoxic effect on tumor cells, as gemcitabine has no direct effect on T-cell proportions in pancreatic patients' blood (Plate et al., 2005). Consistent with this study, we also did not find any significant effect of gemcitabine on tumor-infiltrating and peripheral blood lymphocytes. However, gemcitabine may also yield changes to the tumor cells, which improve the ability of other immune cells to mediate cell death. A low dose of gemcitabine has been shown to upregulate NKG2D ligand expression on tumor cells, which increase the cytotoxic activity of NK cells (Zhang et al., 2020). These gemcitabine effects was only observed on tumor cells, with no direct effects of gemcitabine on Ki67, NKG2D, and IFN-γ expression on purified splenic NK cells (Zhang et al., 2020). In this study, we measured splenic NK cytotoxic activity against PDAC4662 cells that were not treated with gemcitabine *in vitro*. We may therefore have missed a difference in NKCA between gemcitabine-treated mice and non-gemcitabine treated mice. In future, we recommend measuring NK cell function against tumor cells more closely mimicking the *in vivo* condition, such as including chemotherapy in cultures if chemotherapy was provided to the animals.

5.2 Effect of exercise training interventions on pancreatic tumor growth and immune system in mice without gemcitabine

The existing literature has more frequently focused on the effects of exercise on tumor growth in mice without the addition of chemotherapy. This has important

ramifications for the translation of these studies to the clinical world, where most patients are provided with chemotherapy. In studies where exercise has been shown to lower tumor growth, it is unknown if a similar benefit would remain with the addition of chemotherapy. We begin to speak to these potential differences in exercise effects with and without chemotherapy in our current study, as we also compared the effects of the three different exercise interventions on tumor growth in mice not provided with gemcitabine treatment.

5.2.1 Exercise effects on pancreatic tumor growth in mice without gemcitabine treatment

In experiment without gemcitabine, we found a significant difference in tumor growth between groups. This overall group effect was likely due to Cont-LV, which exhibited a trend for significantly slower tumor growth than Sed and HIIT. Interestingly, Cont-LV also had a significantly greater proportion of CD8+T-cells within the tumor. As CD8+ T-cells have been shown to have potent anti-tumor potential, the increased proportion of these cells within Cont-LV tumors may explain the reduced tumor growth.

Many studies have investigated the effects of exercise training on tumor growth and incident in animal models where additional treatments are not provided. The results are inconsistent. Malicka and colleagues have reported a decrease in the number of mammary tumors in mice with exercise, an effect which directly relates to exercise intensity (Malicka et al., 2015). A study that used a HIIT intervention (treadmill running at 15 m/min for 2 minutes followed by 2 minutes of rest, 60 minutes 3 days/week for 4 weeks) found remarkably reduced mammary tumor growth in obese mice (Barra et al.,

2017). Further, Zhang and colleagues reported seven weeks of moderate-intensity swimming exercise for just 8 min 5 days/week significantly reduced tumor growth in a mouse model of liver cancer (Zhang et al., 2016). In contrast, in an allogeneic subcutaneous lymphoid mice tumor model, Zelinsky and colleagues reported no difference in absolute tumor volume after two weeks of strenuous exercise (20-40m/min at 5% grade for 3h or until fatigue) that was performed at the same time as tumor development, although peak tumor volume in the exercise group occurred two days after the control group (Zielinski et al., 2004). Woods and colleagues also found no differences in mammary tumor growth or overall incidence in mice following either "moderate" or "exhaustive" exercise prescriptions compared to sedentary mice (Woods et al., 1994). Our current results add to this body of literature, as we do demonstrate an overall effect of exercise, where tumor growth was significantly reduced. We were not able to detect a significant difference between groups, likely due to the small sample sizes. However, it is interesting that the greatest reduction in tumor growth appears to be in the group that performed the lowest volume of exercise (15 min, 5 days/week) with moderate intensity, contrasting the results of Malicka et al and Barra et al, discussed above. We suggest that this finding may be an important result, as it may be easier for pancreatic patients to complete smaller bouts of exercise. We recommend that these experiments be repeated with a greater sample size.

5.2.2 Exercise effects on immune system in mice without gemcitabine treatment

Understanding immune activity within the tumor microenvironment is important. On the one hand, immune activity may be harmful, as chronic inflammation has been

linked with tumorigenesis while at the same time immunosuppressive, regulatory immune cells are promoted within the tumor preventing anti-tumor immune activity (Binnewies et al., 2018). On the other hand, infiltrating cytotoxic immune cells have been demonstrated as positive prognostic factors for disease outcome and overall survival in several cancers (Binnewies et al., 2018). The isolation of tumor-specific cytotoxic T lymphocytes (CTL) cells from the peripheral blood or tumor tissue of patients across a variety of cancers such as melanoma and lung carcinoma provided evidence of the existence of CD8 T-cell mediated anti-tumor immunity (Boon et al., 1997; Echchakir et al., 2000; Karanikas et al., 2001; Slingluff et al., 1994; Weynants et al., 1999). The existence of a tumor-specific CTL response was further strengthened by identification of tumor-associated antigens (TAA) and detection of TAA-specific CD8+ T-cells in spontaneously regressing tumors (Boon et al., 1997). Studies have shown that T-cell infiltration, especially CD8 T-cell into the tumor microenvironment, correlated with better prognosis in multiple malignancies such as breast, lung, and melanoma and brain cancer (Fridman et al., 2012; Fu and Jiang, 2018; Pagès et al., 2005; Reiser and Banerjee, 2016). In pancreatic cancer patients, tumor-infiltrating CD8+ T-cells together with CD4+ T-cells served as a favorable prognostic factor, and high densities of CD8+ T-cells in the juxta-tumoral area showed better survival in patients with pancreatic cancer (Ene-Obong et al., 2013). In mice, one study has shown that exercise suppresses tumor growth by increasing the infiltration of immune cells such as NK cells and T-cells in tumor (Pedersen et al., 2016). In this study, we found that continuous low volume exercise training increased the infiltration of CD8+ T-cells in tumors. We did not find any increase in tumor-infiltrating NK and CD4+ T-cells, nor any change in peripheral blood lymphocytes (NK, CD4+, and CD8+ T-cells). Importantly, we

found a significant negative correlation between CD8+ T-cell proportions within the tumor and tumor volume. Our results, combined with those previously reported in the literature, suggest that CD8+ T-cells have a predominant role in controlling tumor growth in Cont-LV mice. Future work should focus on understanding the mechanisms driving CD8+ T-cell infiltration into the tumor with exercise.

We did not expect to find a significant effect of Cont-LV on immune cells only, but rather hypothesized greater effects in Cont-HV and HIIT on tumor infiltrating and peripheral blood lymphocytes and NK cell function. Previously, Zelinsky and colleagues, using a long duration high intensity exercise intervention (20-40m/min at 5% grade for 3h or until fatigue) reported an association between tumor regression and increased numbers of tumor-infiltrating lymphocytes at peak tumor occurrence, although the effect was not significant (Zielinski et al., 2004). Barra and colleague reported increased number and activity of circulating NK cells in obese breast cancer mice after 4 weeks of HIIT (Barra et al., 2017). We therefore expected to see increased immune cells and activity in our Cont-HV and HIIT groups. However, it may be that our exercise intervention did not last long enough to see an effect, as the study by Barra et al was twice as long as those described here. Zhang and colleagues also have shown that moderate swimming exercise of just 8 min, 5 days/week initiated three weeks before tumor cell inoculation and continue for seven weeks enhanced CD4+ and CD8+ T-cells in peripheral blood and reduced CD4+ CD25+ Foxp3+ Treg proportion in PBMC, spleen lymphocytes, and TIL (Zhang et al., 2016). We did not find any effect of exercise in peripheral blood lymphocytes, again perhaps due to differences in the length of the intervention. We recommended using longer exercise intervention for future studies.

6. STRENGTHS AND LIMITATIONS

To our knowledge, this is the first study comparing the effects of the three different exercise interventions initiated after tumor development on tumor growth with and without gemcitabine. This study has several strengths. First, we attempted to design a study that better reflects the clinical scenario than many animal models of cancer have done previously. We did this by providing gemcitabine treatment alongside exercise. We also attempted to select exercise volumes and intensities that might be achieved in pancreatic cancer patients (Ngo-huang et al., 2017; Parker et al., 2018). For example, it is hard to imagine many patients being able to perform daily runs lasting 3hours (as modeled in the work by Zielinksi and colleagues). A further strength of this study is the inclusion of a set of experiments examining the effects of exercise without gemcitabine treatment, as this allows some comparisons to the existing literature. Finally, this study included experiments designed to investigate potential mechanisms underlying the effects of exercise on tumor growth, as we measured lymphocytes in tumors and blood. We believe that these study results will helpful to guide future studies.

There are also several limitations of this study. First, we did not measure any fitness markers of mice. Therefore, we do not know how the different exercise interventions impacted the animals' cardiorespiratory fitness. It would be interested to understand if fitness relates to the tumor outcomes measured here. We also do not now how the gemcitabine treatment impacted the animals' ability to exercise. Our assumptions of exercise intensity were based on work performed in healthy mice. It may be that in tumor bearing mice treated with chemotherapy, the 'moderate' intensity would occur at a slower speed. In this study, we only included aerobic exercise interventions. There is

evidence that resistance training also improves patients' quality of life. In the future, resistance training should also be included in exercise oncology studies. Along these lines, it would also be interesting for future experiments to include some indication of quality of life. Our sole indicator was body weight. It has been reported that animals undergo three phases of weight change during tumor challenge; an initial period of weight loss associated with irradiation and tumor implantation, a period of weight gain when the animals appeared to be in a clinically stable condition, and a terminal period of weight loss that preceded death (Toth, 2000). In our study, animals did not lose weight, suggesting that the mice were clinically stable throughout the experiment. Irreversible sudden weight loss or marked decrease in weight during an exercise intervention may also be an indicator of cachexia (Redgate et al., 1991) and overtraining (Kadaja et al., 2010). As we did not see any weight change during the interventions, it is likely that the mice did not lose significant muscle mass and that the exercise protocols used in this study are not overly ambitious.

Another limitation to our study is that the mice completed the forced treadmill exercise during the 12h period of the day with light. Mice are nocturnal animals and naturally most active during early night (no light). Thus, the mice were awakened from their sleep cycle/lower activity period to run. As discussed earlier, forced treadmill running in itself may induced a stress response, with increased anxiety and increased levels of corticosterone (Svensson et al., 2016). Thus, our protocol may have induced several stressors on the animals, which might affect immune system and thus tumor outcomes. We did control for some of these potential stressors, by also manipulating mice in Sed and Sed+Gem (that is, moved their cage close to treadmill, handled the mice). In

the future, we recommend measuring stress hormones, and running the animals as close to their natural awake cycle as possible.

One another limitation of present study is the development of ulcers within tumors. Ulcerated tumor may continuously exude body fluids and may result the acute immune attack (Zhang et al., 2018). In the present study, we euthanized mice early to avoid immune changes generated due to ulcer. However, we do not know that if it already changed the tumor microenvironment. In future studies, we recommended to inject tumor cells in mammary fat pads. Mammary fat pads' surrounded with endogenous support cells that form a biological barrier against contact with the skin, which may have prevented ulceration (Zhang et al., 2018).

There are also many more facets of the immune system that are important in understanding tumor control. For example, as discussed above, MDSCs, tumor associated macrophages, and regulatory T-cells are associated with poor tumor outcomes. We unfortunately did not measure these in the current study.

7. CONCLUSIONS AND FUTURE DIRECTIONS

This study assessed the effects of three different two-week long exercise interventions on pancreatic tumor growth in mice and reported no effect of exercise training on tumor growth with chemotherapy. However, there was a significant difference in tumor growth between the groups without gemcitabine treatment. In these experiments, Cont-LV animals had a trend for significantly lower tumor growth compare to Sed and HIIT. This reduction was accompanied by an increase in the proportion of CD8+ T-cells within the tumor. In future studies, we recommended using longer exercise training interventions. The effect of exercise timing relative to tumor growth is also an important factor that needs to be explored in future studies.

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