Infiltrating and Resident Myeloid Cells in the Central Nervous System are Neuroinflammatory Mediators of Cachexia during Pancreatic Cancer

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Abstract

Cachexia is a devastating syndrome with cardinal features of anorexia, weight loss, and fatigue. This syndrome occurs during numerous chronic diseases, such as cancer, cirrhosis, congestive heart failure, and rheumatoid arthritis. While mechanisms of cachexia are still not well understood, our lab and others demonstrated that inflammation within distinct brain regions can cause signs and symptoms similar to those observed during cachexia. Specifically, inflammation in the mediobasal hypothalamus can drive aberrant activation of the weight- and activity-mediating neurons in the region, causing decreased food intake, loss of skeletal muscle, and decreased locomotor activity. Mechanisms by which inflammation generated in the periphery is translated to inflammation in the brain have yet to be elucidated. Furthermore, cellular sources of neuroinflammation during cachexia are not known. The purpose of this dissertation was to identify and characterize the role of immune cells in the brain during cancer cachexia.

We observed that, in a mouse model of pancreatic ductal adenocarcinoma (PDAC)-associated cachexia, thousands of circulating myeloid cells infiltrate the brain. The majority of these cells were neutrophils, which accumulated at a unique CNS entry portal within the meninges called the velum interpositum (VI). A large percentage of neutrophils in the VI expressed CCR2, an atypical granulocyte receptor. CCR2 knockout (CCR2KO) mice had attenuated cachexia during PDAC and a 40% reduction in total brain-infiltrating neutrophils, along with a 90% reduction of VI-infiltrating neutrophils. There were no differences in circulating, liver, or tumor-infiltrating neutrophils in CCR2KO tumor mice compared to WT tumor mice.

Intracerebroventricular injection of oxidized ATP, a P2RX7 antagonist, prevented neutrophil recruitment to the brain and attenuated cachexia during PDAC. RNASeq analysis of circulating, liver, tumor, and brain neutrophils during PDAC revealed a distinct transcriptional profile in brain-infiltrating neutrophils, indicative of degranulation and release of cytotoxic proteins.

In addition to neutrophil infiltration, we observed microgliosis and astrocytosis, specifically in the mediobasal hypothalamus, throughout the course of PDAC cachexia. Microglia depletion worsened cachexia during PDAC, as indicated by increased anorexia, fatigue, and muscle catabolism. Microglia responded to tumor-derived factors *in vitro*, by producing high levels of transcript coding for the anti-inflammatory enzyme arginase-1 (*Arg1*). We also observed *Arg1* induction in the hypothalamus *in vivo* during PDAC. These results suggest microglia are protective against cachexia during PDAC.

These results demonstrate that brain-infiltrating myeloid cells generate cachexia while resident myeloid cells are productive against cachexia. Further studies, to identify cachexia-inducing factors produced by brain-infiltrating neutrophils, along with neuroprotective factors produced by microglia, are necessary.

Introduction

Overview

The topic of this dissertation is cachexia, a chronic disease-associated metabolic syndrome. In order to understand processes important for cachexia, one must first understand sickness behaviors. As such, Section 1 of the Introduction provides a background on sickness behaviors. In addition, Chapters 1 and 2 are published review papers and therefore provide a thorough review of literature pertaining to many of the concepts covered in this dissertation. As such, Sections 1 and 2 of the Introduction, which introduce the content covered in Chapters 1 and 2, are abbreviated to avoid redundancy. Sections 3 and 4 provide more extensive review of literature of their respective topics, as they are not covered in review papers elsewhere in this dissertation.

1. Sickness Behaviors

When an organism is challenged by a pathogen, a highly conserved and highly coordinated metabolic program is initiated, wherein the organism diverts energy away from certain processes in order to combat infection. The main features of this process include fever, tissue breakdown, anorexia, and decreased locomotor activity (1). Other symptoms, including depression (2), cognitive dysfunction (3), and anxiety (4), are also present. This constellation of signs and symptoms is collectively referred to as "sickness behaviors" (5). Sickness behaviors occur in nearly all animals (6), and the resulting

alterations in metabolism are important for surviving infection (7). All sickness behaviors can be explained in terms of a beneficial response for combating infection. For example, fever produces an environment unfavorable to pathogens (8), yet favorable for immune cell activity (9) and mobilization (10). The benefits of anorexia are still not fully understood (11), but studies show that during acute infection force feeding animals decreases survival (12). It was also hypothesized that anorexia occurs in order to decrease foraging behavior so as to minimize activity and reduce availability of nutrients, particularly iron, to pathogens (13). The same can be said about fatigue, which is thought to prevent organisms from using energy for locomotor activity (14). Lastly, tissue catabolism occurs in order to mobilize protein to fuel energy-expensive immune cells (15).

There is an extensive amount of research on the relationship between immune activation and sickness behaviors. Since components of viruses, bacteria, parasites, and fungi activate the immune system (referred to as pathogen-associated molecular patterns, or PAMPs), it was hypothesized that immune system activation can induce the prototypical signs and symptoms of sickness behaviors (16). Indeed, administration of PAMPs such as bacterial lipopolysaccharide (LPS) (17) and the viral double stranded RNA mimetic Poly I:C (18) induce sickness behaviors. Robert Dantzer's lab's work was instrumental to the current understanding of relationship between immune activation and sickness behaviors (2, 5, 19), especially the concept that the brain can sense signals associated with infection and induce sickness behaviors (reviewed in detail in Chapter 1). Therefore, many studies from his lab will be described. Since the brain controls many of the processes involved in sickness behavior (appetite,

wakefulness/locomotor activity, cognition, etc), it was hypothesized that the immune system is able to communicate the presence of an infection to the brain in order for the brain to change the body's metabolism accordingly (20).

Much of this work focused on the key innate immune cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF). It is well documented that administration of both IL-1β and TNF-α directly into the brain induce sickness behaviors (21-23). Moreover, IL-1β can directly activate certain neuron populations in areas important for sickness behavior (24, 25), leading to secretion of hormones and neurotransmitters that can influence behavior. In addition, systemic administration of LPS induces inflammatory cytokine expression in the CNS (26-28), suggesting the brain is not only a receiver of inflammatory signals, but is also able to respond, amplify, and modify the message it receives.

While sickness behaviors represent an important clinical concern, most acute infections are cleared within days and symptoms resolve as the infection is cleared. This is a product of a conserved, coordinated process that developed over thousands of years of organisms challenged by pathogens. However, a new set of diseases, usually not directly caused by pathogens, has emerged, due to increased lifespan and altered diet. These diseases include heart failure, cancer, rheumatoid arthritis, chronic obstructive pulmonary disease, kidney failure, cystic fibrosis, acquired immunodeficiency syndrome, and cirrhosis. While these diseases are chronic in nature, an immune response similar to pathogen challenge occurs, initiating a process similar to sickness behaviors. Unlike acute infection, the inflammatory instigator is not cleared,

preventing resolution. When this process is continued, it becomes maladaptive, developing into a devastating syndrome called cachexia

2. Cachexia: A chronic disease-associated metabolic syndrome

Cachexia is a chronic disease-associated metabolic syndrome with cardinal features consisting of muscle and fat loss, anorexia, and fatigue. The term cachexia is derived from the Greek words *kakos* "bad" and *hexis* "habit" Although descriptions of weight loss, anorexia, and fatigue date back to King David nearly 3,500 years ago, the term cachexia was likely first described by the Greek philosopher Celsus, in his writings on different categories of "consumption", sometime between 25 B.C. and 50 A.D.: "…There is a second species, which the Greeks call cachexia, where the condition of the body is bad: and on this account all the nutriment becomes putrid (29)." Cachexia was long discussed as a serious clinical concern, yet is still a poorly defined and inadequately addressed entity, despite recent efforts to develop consensus definition (30, 31). The current international consensus definition for cachexia, defined in 2011, is: "….a multifactorial syndrome characterised by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. The pathophysiology is characterised by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism." The diagnostic criteria for cachexia are: weight loss greater than 5%, or weight loss greater than 2% in individuals already showing depletion according to current body weight and height (body-mass index [BMI] <20 kg/m^2)) or skeletal muscle mass (sarcopenia) (30).

Cachexia is estimated to occur in over 150,000 hospital patients each year, and is associated with increased length of stay and loss of function (32). Moreover, it is associated with poor outcomes in several chronic diseases, including congestive heart failure (33), rheumatoid arthritis (34), chronic pulmonary obstructive disease (35), human immunodeficiency virus (HIV) (36),and cancer (37, 38). Out of these diseases, cancer receives the most attention. Cachexia occurs in up to 80% of cancer patients, and is associated with decreased survival (39), decreased quality of life (40), and decreased tolerance to chemotherapy (41). Since the topic of this dissertation is cancer cachexia, the remainder of this review will focus on literature related to cancer-associated cachexia. Specifically, this dissertation focuses on pancreatic ductal adenocarcinoma (PDAC), a deadly malignancy of the exocrine pancreas with a high rate of associated cachexia. The 2015 median five year survival rate of patients with PDAC was estimated at approximately 7% (42), and the median survival after diagnosis is as low as 6 months (43). PDAC is projected to become the second leading cause of cancer death in the U.S. by 2030 (44). Despite the devastating nature of this disease, survival has not significantly improved over last several decades, and treatments are still lagging behind those for other cancers.

Therapies for PDAC largely consist of: 1) combination therapy with the nucleoside analogue, gemcitabine, and the microtubule inhibitor abraxane, 2) folfirinox, which consists of four different agents (a platinum-based agent, a pyrimidine analogue, a topoisomerase inhibitor, and a vitamin B9 derivative to reduce the side effects of the pyrimidine analogue), and 3) surgery (45). While folfirinox is more effective than gemcitabine in metastatic (50% of PDAC patients are diagnosed with metastatic

disease) and surgically resected PDAC (46, 47), chemotherapies for PDAC are much less effective than those for other malignancies. Surgery is the only potentially curative treatment, yet five year survival rates after resection alone are low (10% or less), and the majority of PDAC patients are not surgical candidates at diagnosis (48). One of the main reasons for these poor outcomes is the fragility of patients with PDAC. This limits their ability to tolerate sufficient doses of toxic chemotherapies and prevents them from being candidates for surgery.

It is important to distinguish cachexia (a metabolic syndrome) from primary anorexia or starvation, wherein during the latter skeletal muscle is largely spared until prolonged food deprivation (49). Alternatively, during cachexia skeletal muscle catabolism occurs early in the disease process (50). Moreover, metabolic profiling of several different tissue types during cachexia revealed a distinct profile compared to that during starvation (51, 52). The identity of cachexia as a true metabolic syndrome, rather than one of starvation, became even more evident when studies investigated the potential of forced nutrition, via total parenteral nutrition, to ameliorate cachexia. These studies revealed that intravenous nutrition could not reverse cachexia symptoms (53, 54). It is worth noting that, like most clinical cachexia studies, there was a small number of participants, with substantial heterogeneity. However, other studies assessing the efficacy of nutrition during cancer cachexia demonstrated limited benefit from various nutritional interventions (55). These studies indicate that metabolic dysfunction is a key aspect of cachexia, and preventing this dysfunction is critical for treatment. In order to correct metabolic dysfunction, its cause must first be understood. A common underlying

feature of cancers with associated cachexia is inflammation, which is proposed to modulate metabolism.

2.1 Inflammation in cancer cachexia

Despite disagreement as to exact mechanisms of cachexia, most researchers agree that inflammation is a key component (56-58). In general, systemic inflammation is associated with poor prognosis during various types of cancer (59-61). One of the major roadblocks for therapy development is the lack of consensus on the definition of "systemic inflammation". Inflammation can be viewed at the cellular level (i.e., infiltration of circulating immune cells into tissue) or at the molecular level (i.e., production of inflammatory mediators such cytokines, prostaglandins, toll-like receptor agonists, etc.). Regardless, several studies showed that during cancer, there is increased serum concentrations of C-reactive protein (CRP) (62), IL-6 (63), IL-1β (64, 65) , TNF-α (66). At the cellular level, it was shown that leukocytosis (67) and increased neutrophil to lymphocyte ratio (NLR) (68) occur during cancer.

Increased levels of circulating IL-6 (69), TNF-α (70), CRP (69), and an increased NLR (71) are all associated with cachexia. As such, several reviews recommended blockading inflammatory mediators to mitigate cachexia (56, 57, 72-74). However, very few preclinical studies assessed the efficacy of cytokine blockade in cancer cachexia. Almost all investigated IL-6. One study demonstrated decreased cachexia in a mouse model of colon cancer after administration of a small molecule IL-6 inhibitor (75). In animals inoculated with tumors that overexpress IL-6, blockade of IL-6 attenuates cachexia (76, 77). The circular logic involved in these studies is obvious, limiting their usefulness. One study administered an IL-6 neutralizing antibody to tumor-bearing nude

mice and reported decreased weight loss (78). The use of immunodeficient mice in tumor studies is declining significantly with increased appreciation of the immune system's role in tumor development. As such, the utility of tumor studies in nude mice is limited. Lastly, IL-6 blockade was reported to normalize liver ketogenesis (important to support the brain's energy demands) during PDAC in mice (79).

In humans, clinical trials involving systemic inhibition of various inflammatory mediators during cachexia showed mixed results. For example, thalidomide, which decreases TNF-α synthesis, showed benefit in preventing weight loss during pancreatic cancer (80). However, larger multicenter clinical trials assessing the efficacy of infliximab, a monoclonal antibody against TNF-α, in treating cachexia during pancreatic cancer, did not demonstrate any differences between treatment and placebo groups (81). In other types of cancer this treatment caused worse outcomes (82). The same was true for a trial assessing the effects of etanercept, a TNF-α decoy receptor, during cachexia in patients with various cancer types. Patients receiving etanercept showed no improvement in weight gain, survival, or subjective symptoms versus those receiving placebo. Moreover, etanercept treatment was associated with a large increase in neurologic complications (29% vs. 0% in placebo group) (83). However, in a trial assessing the effects of different combinations of chemotherapy and immunomodulators on cachexia in different types of cancer, which involved a combination of medroxyprogesterone, megestrol acetate (an agent shown to improve appetite, which will be discusses in the next section), oral amino acid and omega-3 fatty acid supplements, and thalidomide demonstrated improved appetite, systemic inflammation, and subjective prognostic score (84). Because of these conflicting results and the

complex nature of proposed therapeutics, there are currently no FDA-approved cytokine blockade therapies for cachexia.

In addition to inflammatory cytokines, several studies investigated the role of cyclooxygenase (COX) inhibitors and other nonsteroidal anti-inflammatory drugs (NSAIDs). COX is important for generation of prostaglandins and other inflammatory mediators. In mice, administration of the NSAID Ibuprofen attenuated neuroinflammation and fatigue in a mouse model of colon cancer (85). In another mouse model of cancer cachexia, administration of indomethacin, a nonselective inhibitor of both COX 1 and COX 2, abrogated cachexia (86). However, in this study, antibody-mediated or genetic blockade of prostaglandin E2 (downstream of COX) did not attenuate cachexia, suggesting additional research is needed to identify the mechanism of COX-mediated inflammation in cachexia. Nevertheless, in a small clinical study of 135 patients with various types of advanced cancers, patients receiving indomethacin survived double the number of days than those in the placebo group (250 days in placebo group vs. 510 days in indomethacin group) (87). This is not yet replicated in a large scale clinical trial.

Despite advances in defining molecular mediators of cachexia, the key cellular source(s) of inflammation during cancer cachexia is/are still not known. Studies show that tumor removal can attenuate or even eliminate cachexia (88, 89), suggesting that factors inducing cachexia are tumor-derived or tumor-associated. However, these models were all subcutaneous tumors, which do not recapitulate the tumor microenvironment, and for many patients tumor removal is not possible. Furthermore, the lack of consistency and success of anti-inflammatory clinical trials suggests our

knowledge of this syndrome is incomplete. Moreover, the effects of circulating inflammatory mediators on different organs, and how this is translated into inflammatory mediators produced within the organ itself, are still not well understood. The next section will describe how the brain is able to sense and generate inflammation in response to an inflammatory insult in the periphery, and how this can cause cachexia.

2.2 Central Nervous System Mechanisms of Cachexia

The concept that the brain is able to control various aspects of metabolic function (feeding behavior, tissue breakdown, etc.) was introduced briefly in Section 1. In mouse studies and a few human studies (both discussed below) there is evidence that neuronal properties and activity are altered during cancer cachexia. This section will highlight studies investigating how the brain mediates cancer cachexia. First, the hypothalamus will be introduced, due to its role as the master regulator of energy homeostasis.

2.2.1 The Hypothalamus is the Master Regulator of Energy Homeostasis and is Dysfunctional during Cancer Cachexia

The hypothalamus is located at the base of the brain, adjacent to the third ventricle. This region consists of over 50 nuclei, which contain neurons that control a plethora of homeostatic functions including appetite, sleep, activity level, wakefulness, body composition, and aging. Due to their importance in processes disrupted in cachexia, the arcuate nucleus and median eminence will be the main focus of this section. The arcuate nucleus of the hypothalamus is considered the body's command

center for controlling energy balance (90). This nucleus contains well-studied neuronal populations that control appetite and other homeostatic processes. When Pro-opiomelancortin/cocaine and amphetamine-regulated transcript (POMC/CART) neurons in the arcuate nucleus are activated, feeding behavior decreases (91), a process that is dependent on melanocortin receptor signaling. Alternatively, stimulating a separate population of neurons in this region, which express agouti-related peptide (AgRP) and neuropeptide Y (NPY), is sufficient to elicit feeding (91). Insulin secreted by the pancreas increases POMC expression (decreasing appetite), while decreasing AgRP neuropeptide expression (also decreasing appetite). Leptin, secreted by adipocytes, acts similarly on AgRP and POMC neurons, also decreasing appetite (92). Alternatively, ghrelin, which is secreted by cells within the gastrointestinal tract, has the opposite effects of leptin and insulin, increasing AgRP neuron activity and decreasing POMC neuron activity, stimulating appetite (93). These neuropeptides are important for hypothalamic regulation of feeding behavior, and implicated in certain diseases processes, but are not the focus of this dissertation and will therefore only be discussed briefly hereafter.

Adjacent to the arcuate nucleus is the median eminence (ME), a circumventricular organ (CVO) that lacks a blood brain barrier (BBB) and contains fenestrated capillaries. Corticotrophin Releasing Hormone (CRH), Gonadotropin releasing hormone (GnRH), and Thyrotropin Releasing Hormone (TRH) neurons all send projections to the fenestrated capillaries of the ME, where they secrete hormones into the portal system, which act on the pituitary gland to stimulate additional hormone secretion (94).

It is well established that the ME lacks a BBB and is therefore accessible to many circulating factors and metabolites. The accessibility of the arcuate nucleus (and therefore POMC and AgRP neurons) to circulating factors is an active topic of debate. It is generally considered that under non-pathologic conditions, the arcuate nucleus lies within the BBB. However, in fasting animals, the BBB is modified by tanycytes (specialized ependymal cells lining the third ventricle floor) and the arcuate nucleus becomes freely accessible to many circulating factors (95). Similar effects happen during obesity (96), but the effects of a peripheral cancer on BBB permeability within the hypothalamus has yet to be assessed.

Few studies directly assessed the effects of cancer on hypothalamic neuron activity. It was shown that genetic (via deletion of the gene for the type 4 melanocortin receptor [MC4R]) or pharmacologic disruption (via ICV administration of AgRP) of the central melanocortin system completely abolished cachexia in mice with cancer (97). The efficacy of AgRP and small molecule MC4R antagonists in ameliorating cachexia was confirmed in additional rodent cancer models (98). Early stage clinical trials of MC4R antagonists in cancer cachexia are currently in progress. In addition to the POMC, increased melanin-concentrating hormone receptor immunohistochemistry staining in the infundibular nucleus in humans with cachexia (99). Interestingly, increased NPY expression is correlated with anorexia in mouse models of cachexia (100). Furthermore, a recent study in humans by Molfino et al. (101) demonstrated decreased hypothalamic activation (via BOLD fMRI) after visualizing a food stimulus in patients with lung cancer-associated anorexia. However, this study was very small (n=4 in the non-anorexic group and n=9 in the anorexic group) and did not make adjustments

for signal background. These studies suggest the hypothalamus is dysfunctional during cachexia, and further research is necessary to determine mechanisms by which this occurs.

In addition to the hypothalamus, other brain regions are implicated in energy homeostasis dysfunction during cachexia. For example, the lateral parabrachial nucleus, located in the brainstem, is implicated in control of feeding behavior (102, 103). A recent study utilizing the Lewis lung carcinoma model of cancer cachexia showed that inactivation of calcitonin-gene related peptide neurons in the lateral parabrachial nucleus prevented cachexia (104). However, several questionable findings were presented, including extremely robust c-Fos staining in the setting of chronic neuronal activation (c-Fos is a marker of acute neuronal activation and usually disappears shortly after initial activation), a remarkably anorexigenic Lewis lung carcinoma clone (in our hands, the Lewis lung carcinoma tumor line is mildly anorexigenic at best), and unusual calorimetry readings. Nevertheless, this study presents a novel neural circuit possibly dysfunctional during cancer cachexia.

In addition, the area postrema (AP), located at the base of the fourth ventricle, is referred to as the brain's "vomit center". Like the hypothalamus, the AP contains POMC neurons and fenestrated capillaries. Lesions in this region during neuromyelitis optica, an autoimmune channelopathy, cause intractable vomiting, a syndrome called "area postrema syndrome" (105). Growth differentiation factor 15 (GDF-15) is shown to act on its receptor GDF-15 receptor alpha-like (GFRAL), which is expressed exclusively in the AP, to promote anorexia and weight loss (106). It was also shown that supraphysiologic levels of GDF-15 induce cachexia (107), and GDF-15 blockade

attenuates cachexia (108, 109). These recent studies present an exciting avenue for potential cachexia treatments. Further investigations are necessary to understand the neural circuits involved in GDF-15 signaling-induced anorexia.

2.3 Current cancer cachexia therapies are not effective

Currently, there are only two treatments used clinically for cachexia, both of which have limited utility and are FDA approved only for AIDs-associated anorexia and weight loss. Both of these treatments are thought to act on the brain. Dronabinol is a synthetic tetrahydrocannabinoid, and megestrol acetate is a progesterone derivative (110). Megestrole acetate is believed to stimulate neuropeptide Y release, thereby improving appetite, but its mechanism is still not fully understood. In clinical trials for cachexia, this drug slightly improved appetite but did not improve quality of life or survival (111, 112). Dronabinol is thought to also stimulate appetite. A study including 289 patients with advanced cancer comparing appetite in patients that received either a cannabis formulation or placebo was terminated early because there were no observed differences between treatment arms (113). In a study comparing dronabinol to megestrole acetate treatment during cachexia in patients with various cancer types those receiving megestrole acetate experienced greater appetite improvement and less weight loss compared to those receiving dronabinol (114). However, a recent meta-analysis of megestrole acetate in cancer cachexia clinical trials showed no increase in quality of life and an increase in adverse events in patients receiving megestrole acetate versus those receiving placebo (115).

In addition to megestrol acetate and dronabinol, anamorelin, an orally active ghrelin analogue, showed promise in preventing weight loss and other cachexia

symptoms in a phase III clinical trial of patients with non-small cell lung cancer-associated cachexia (116). However, this medication is currently not FDA approved and was rejected by the European Medicines Association, due to lack of improvement in handgrip strength and the minor weight gain observed (117).

These failures illustrate the desperate need for effective cachexia therapies. Moreover, they demonstrate that additional research is needed to better understand cachexia mechanisms, especially those that pertain to appetite dysregulation and weight loss. Neuroinflammation is a potential means by which inflammation could be linked to neuronal dysfunction to cause appetite dysregulation and other cachexia symptoms.

2.3 Neuroinflammation as a potential driver of cachexia

The brain's ability to sense and respond to an acute peripheral inflammatory insult was described earlier. There is ample evidence to suggest a similar process occurs in the MBH during cachexia (reviewed in (118-121)). In rats and mice, there is increased expression of inflammatory cytokine transcripts in the MBH during several different models of cancer cachexia (122-125). Aside from cachexia, there is also evidence of depression and anxiety-like behavior occur during cancer in the absence of overt cachexia (126). Several studies show that IL-1β is the major cytokine induced in the MBH as a result of a peripheral tumor (122-124). Outside of cancer, IL-1β is elevated in the CSF of individuals with rheumatoid arthritis (a condition associated with cachexia) compared to those without inflammatory disease and those with multiple sclerosis (127). Taken together these reports implicate brain-derived IL-1β in the

pathogenesis of cancer cachexia.   However, Grossberg et al. recently showed that deletion of IL-1β did not attenuate fatigue in several different rodent models of cancer cachexia (128). It is worth noting that anorexia was absent from all cancer models tested in this study, raising the question as to whether the models used were accurate representations of cancer cachexia.

Alternatively, the adaptor protein myeloid differentiation factor 88 (MyD88), which is a key signaling mediator for the IL-1 receptor and several TLRs, is implicated in the pathogenesis of cancer cachexia. In two separate studies using different models of cancer cachexia, MyD88 deletion attenuated several measures of cachexia, including anorexia, muscle catabolism, fat loss, hypothalamic inflammation, and fatigue (129, 130). These results directly contrast with Grossberg et al., which reported that MyD88 deletion did not attenuate fatigue during cancer cachexia (128). In an effort to identify the key cell type involved in MyD88-driven cachexia, Ruud et al. deleted MyD88 from myeloid cells (using the $Mx1^{Cre}$ driver) and observed decreased anorexia and weight loss in sarcoma-bearing animals (131). While the authors made the claim that these results indicate that MyD88 signaling in hematopoietic cells, rather than brain-resident cells, is required for cachexia, $Mx1$ is expressed in microglia to some degree (132).

In the studies mentioned above, MyD88 deletion did not entirely mitigate cancer cachexia, suggesting that other inflammatory mediators are involved. Another important protein for cytokine and chemokine synthesis is the adaptor TIR-domain containing adaptor inducing interferon-β (TRIF). TRIF is traditionally considered key for TLR3 signaling, but is also downstream of several other TLRs. TRIF deletion attenuates acute sickness response after administration of Poly I:C (133), and is involved in TLR4 driven

pathological response to CNS damage (134), but has not been investigated as a potential mediator of cancer cachexia. Chapter 3 will describe a series of studies demonstrating that TRIF is a key inflammatory mediator of acute sickness response and cancer cachexia.

The conflicting results of anti-inflammatory therapies in cachexia, as well as the conflicting results on the role of different cytokines in cachexia suggest that knowledge on mechanisms of hypothalamic inflammation-driven cachexia is far from complete. Cellular sources of inflammation in cachexia, particularly those in the brain, are not yet identified. As described below, resident brain macrophages become activated and are contributors to acute sickness behavior. Therefore, these cells present a potential cancer cachexia mediator.

3. Brain macrophages: Potential key players in neuroinflammation and cachexia during chronic systemic inflammation.

The brain possesses a diverse immune cell network, which participates in several homeostatic and pathologic functions, albeit in a substantially different manner than that which occurs in other organs (135, 136). While other cells (astrocytes, ependymal cells, pericytes, endothelial cells, etc.) are capable of reacting to PAMPs, the major resident immune cells in the brain are macrophages of the myeloid lineage (137-139). These macrophages include: 1) microglia: the most well-known and extensively studied. These are the only immune cells that reside within parenchyma in non-pathological states. 2) perivascular macrophages: in direct contact with vascular endothelium throughout the brain. 3) meningeal macrophages: present in the subdural meninges, in contact with the CSF and meningeal blood vessel lumens. 4) choroid plexus macrophages: the only population of macrophages in the brain that experiences

turnover from circulating monocytes, and are able to sample CSF (140). A series of elegant fate-mapping studies showed that all brain-resident macrophages are derived from embryonic progenitor cells in the pre-hematopoietic yolk sac, which populate the primitive CNS early in development and are maintained via local self-renewal (140, 141). Despite the longstanding belief that brain macrophages are largely quiescent, a recent explosion of studies, particularly on microglia, demonstrated that these cells play a prominent role in both health and disease (142). This section will highlight literature investigating the role of brain resident macrophages in modulating neuroinflammation and as potential mediators of metabolic dysfunction during disease. Only microglia will be discussed, since there is currently minimal literature on non-parenchymal macrophages.

3.1 Microglia

Microglia are the sole resident macrophages of the CNS parenchyma. Until the early 2000's, these cells were considered mainly quiescent except in the context of profound neuropathology (stroke, traumatic brain injury, etc.) (143). However, an explosion of recent studies demonstrated that microglia perform several important functions in both homeostasis and disease, including: 1) modulating neuronal circuits (144), 2) controlling the activity of other cells in the brain such as astrocytes (145) and oligodendrocytes (146, 147), 3) combating CNS infections (148), and 4) modulating metabolic function (149).

There is extensive evidence showing that microglia are both genetically and phenotypically perturbed during most CNS pathologies (150), a notion that most

researchers agree upon. However, there is still intense disagreement about their role in disease. Microglia were implicated both as neuroprotective and drivers of pathology in several neurologic diseases, including stroke (151), Alzheimer's disease (152), multiple sclerosis (MS) (153, 154), prion disease (155, 156), and Parkinson's disease (157). These conflicting results indicate that the function of microglia is likely context-dependent, yet still poorly understood, and that further work is necessary to fully understand their role in homeostasis and pathology.

The hypothalamus is densely populated with microglia, which exhibit a diverse array of phenotypes throughout the different nuclei. In particular, microglia within the ME display a unique phenotype depending on their proximity to the fenestrated capillaries. As highlighted in the excellent review by Kaelin et al. (158), microglia in the external zone of the ME have an amoeboid morphology with very short processes, more closely resembling macrophages that traditional parenchymal microglia. In addition to the hypothalamus, microglia in the pituitary gland, which is connected to the ME via the portal vascular system, are able to cleave terminals of neurosectratory neurons (159).

As early as 1992, it was shown that endotoxin exposure in rats causes microglia ramification and expression of IL-1 in the hypothalamus, which lead the authors to postulate that brain macrophages may be key players in sickness response (26). The first study to demonstrate that macrophage-derived factors are capable of generating sickness behaviors showed that when medium from LPS-treated peritoneal macrophages was injected into endotoxin-resistant mice (ruling out residual LPS as a mediator), it induced weight loss and anorexia, which reversed when medium injections were discontinued (160). Furthermore, deletion of the leptin receptor in myeloid cells

induces hyperphagia and increased food intake (161), suggesting that in addition to sensing PAMPs, brain macrophages are capable of responding to hormones generated in the periphery. One study claimed that microglial TLR2 stimulation resulted in sickness behaviors. The authors argued that after TLR2 stimulation, microglia interact with POMC neurons, which drives anorexia and weight loss. This study had numerous flaws, including questionable TLR2 immunohistochemistry, correlative data rather than mechanistic data (TLR2 was never deleted from microglia), and limited cell specific interventions (162). Despite a wealth of data correlating microglia activation with sickness behaviors, only one study showed that microglia activation or inflammatory signaling is necessary for acute anorexia, fatigue, and weight loss. In this study, the authors injected animals with IP LPS and showed that the resulting neuroinflammation, fatigue, weight loss, and depressive-like behaviors were attenuated by administration of the tetracycline antibiotic minocycline (163). Several studies demonstrate the anti-inflammatory effects of minocycline (164), particularly on microglia (165). However, the anti-inflammatory mechanism of minocycline is still not known, and success in preclinical has not translated into success in humans (166). Furthermore, the *in vivo* effects of minocycline on sickness behaviors and neuroinflammation in this study were moderate at best, suggesting that more precise approaches (e.g., deleting inflammatory signaling mediators from migrolia) are needed to better understand their role in sickness behaviors.

Several studies investigated microglia as mediators of metabolic dysfunction in the context of high fat diet-induced obesity (HFDO) (167, 168), which is reviewed in depth in Chapter 1. Briefly, it was first shown (in rodents) that microglia in the

hypothalamus assume an activated morphology shortly after HFDO is initiated (169). This is associated with increased expression of proinflammatory cytokines, demonstrated both *in vivo* and *in vitro* (in cultured microglia after exposure to saturated fatty acids) (170). It was also shown that microglia depletion results in decreased neuroinflammation, food intake, and weight gain during HFDO (149, 170). Lastly, there is some data to suggest that induction of inflammatory transcripts and gliosis occur in the hypothalamii of obese humans (169, 171, 172).

Outside of HFDO, there is much less literature on microglial response to chronic systemic inflammation. During rheumatoid arthritis (173) (a condition associated with cachexia) and systemic lupus erythematosus (not commonly associated with cachexia, but is associated with cachexia-like symptoms and psychiatric disturbances) (174), microglia assume an activated state and modulate neuronal properties. Furthermore, during necrotizing enterocolitis, a devastating neonatal disease that results in profound neurodevelopmental delays, microglia become activated and drive neuropathology (175). However, very few studies investigated microglia as potential mediators of cancer cachexia. The only study that assessed microglia in the context of an extra-CNS tumor was Norden et al. (176), in which microglia in the cortecies of animals inoculated with the colon-26 tumor line assumed a somewhat activated morphology. This was accompanied by neuroinflammation, fatigue, and depressive-like behaviors (all quite mild), which were slightly attenuated by administration of minocycline. In addition to the study by Norden et al., it was also reported that minocycline mildly attenuated cardiac dysfunction in tumor-burdened mice (177). The unimpressive nature of these results

demonstrate that more robust models and treatment paradigms are needed to determine the role of microglia in cancer-associated signs and symptoms.

These studies indicate that there is still much to be learned about how microglia respond to chronic systemic inflammation. As the sole resident immune cells of the brain parenchyma, these cells are intriguing candidates to study as potential mediators of cancer cachexia. Chapter 5 will describe studies investigating the role of microglia in neuroinflammation and metabolic dysfunction during cancer cachexia.

In addition to microglia, there is now evidence that circulating immune cells play a key role in brain immunity. The next section will describe literature related to interactions between leukocytes and the brain, and how these cells should be considered as potential mediators of cachexia.

4. Infiltrating immune cells in the brain: Unexplored potential players in sickness behavior and cachexia

Circulating immune cells are critical for generating systemic inflammation, and can infiltrate the brain during several different pathological states (135), yet have not been investigated in the context of cancer cachexia. There is growing evidence that circulating leukocytes infiltrate the brain during numerous states of systemic inflammation and stress, such as inflammatory liver disease (178, 179), endotoxemia (180), psoriasis (181), and lupus (174). This section will review literature in which the presence, identify, and function of brain-infiltrating immune cells were investigated during chronic disease.

4.1 Routes of immune cell infiltration into the brain

The brain has long been considered an "immunoprivileged" organ, a term that refers to lack of immune response generated from an experimentally implanted tissue graft (182, 183). The eyes and gonads are also considered immunoprivileged, but the brain's immunoprevilaged status is the most studied (135, 184). Unlike other organs, the brains of humans and mice are almost entirely devoid of adaptive immune cells (185). Furthermore, there is little evidence to show that T-cells perform immune surveillance within the brain parenchyma. This is supported by the fact that the brain parenchyma lacks functional dendritic cells (DC). Activated microglia can express CD11c, a DC marker, but their antigen presenting ability is paltry compared to infiltrating DCs (186). However, the fact that MS patients receiving natalizumab (an integrin inhibitor that prevents T-cells from infiltrating brain) can experience reactivated polyomavirus infections in the brain, resulting in the subcortical white matter disease progressive multifocal leukoencephalopathy (187), suggests there may be some degree of immune surveillance occurring in the brain.

The brain's immunoprevilaged status is thought to be largely due to the BBB, which is a tightly regulated physical barrier that prevents cells and most solutes from crossing into the parenchyma from the circulation. The BBB consists of continuous endothelial cells that line the lumen of blood vessels, pericytes in direct contact with endothelial cells, and astrocyte endfeet. The regulation and role of the BBB in states of inflammation, especially systemic inflammation, is still not well understood. This is due to the fact that the BBB is regionally distinct (e.g., CVOs lack a BBB) and limitations of different techniques to study BBB permeability (188) (e.g., it is still not agreed upon

whether a septic dose of LPS can induce BBB breakdown (189)). Although it was suggested that the BBB must be compromised in order for immune cells to cross into the CNS parenchyma (190), there is evidence that immune cells can cross an intact BBB, in both an antigen-dependent (e.g., MS) (191, 192) and antigen-independent manner (e.g., stroke) (193).

The BBB receives the most attention of the barriers between the CNS and the periphery. However, this is not the only entry into the CNS. The CVOs lack a traditional BBB in that they are lined by fenestrated capillaries lacking astrocyte ensheathment and do not contain many of the typical proteins of BBB vessels (194, 195). The CVOs consist of the ME of the hypothalamus, the choroid plexus, the organ vasculosum of the lamina terminalis, the subfornical organ, and the area postrema. As a whole, the CVOs were implicated as an initial entry portal for immune cells in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS (196). The choroid plexus has received the most attention of the CVOs as a potential gateway into the CNS (184, 197). This organ produces CSF and is therefore continuous with the ventricles and other CSF-filled spaces. Unlike the parenchyma, the ventricles and meninges (discussed in the subsequent paragraph) are not immunoprivileged, in that experimentally engrafted tissue is rejected (183). Moreover, the choroid plexus does contain functional DCs and undergoes immune surveillance by a unique T-cell population (198). Recent studies implicate the choroid plexus as a gateway for both lymphocyte and myeloid cell infiltration into both the inflammed (199, 200) and non-inflammed brain (201).

Despite also containing a CVO (the ME), very few studies investigated the hypothalamus as a potential gateway for immune cell entry to the brain. One EAE study

identified the ME as location of leukocyte infiltration early in the disease process (196). During anxiety, immune cells infiltrate the paraventricular nucleus of the hypothalamus (discussed in detail below) (202). After four weeks of HFDO, myeloid cells infiltrate the ME and arcuate nucleus, where they are thought to participate in obesity pathogenesis (149).

Recently there has been considerable interest in the meninges as a key site of immune activity and regulation within the CNS, as well as a gateway for immune cell infiltration into the brain (203, 204). The meninges are CSF-filled and line the entire CNS, consisting of (in most areas) the dura and underlying leptomeninges (containing the arachnoid layer and pia matter). Unlike the CNS parenchyma, the meninges contain a rich immune environment, consisting of specialized meningeal macrophages, resident neutrophils, memory T-cells, functional DCs, immunomodulatory reticular fibroblasts, monocytes, and regulatory T-cells (136, 205, 206). Within the meninges, there is an extensive network of fenestrated pial vessels that lack astrocyte ensheathment and possibly other components of the BBB (206), which are implicated in circulating immune cell entry (207, 208). Immune cells infiltrate the meninges prior to the CNS parenchyma during various states of inflammation (206). While many of these immune cells ultimately do not enter the CNS parenchyma (most likely due to the impermeable nature of the glial limitans), there is increasing appreciation that inflammation limited to the meninges can still cause profound neuropathology and functional deficits (203). For example, meningitis is associated with poor outcomes, including cognitive impairment, hearing loss, seizures, and developmental delays (in children) (209, 210). This underscores the importance of meningeal immunity in different disease processes.

The recent characterization of a functional lymphatic system in the meninges has completely changed how meningeal immunity (and CNS immunity, for that matter) is viewed (211-214). This system consists of vessels within the dural sinus that express all of the molecular hallmarks of lymphatic vessels, contain lymphatic fluid, and are an avenue for circulating T-cells (213). These vessels drain into the deep cervical lymph nodes and therefore potentially provide a conduit for immune communication between the CNS and periphery. Most of the studies on meningeal lymphatics were performed in mice, but there is evidence of dural sinus-associated lymphatic vasculature in humans (215). These vessels provide key routes for immune cell infiltration into the brain during EAE and a passageway for CNS-derived antigens to access peripheral lymph nodes (212). In addition, this system is impaired in aged mice and loses function during Alzheimer's disease (211).

In summary, the brain parenchyma is an immunoprevilaged site that lacks immunosurveillance and in homeostatic conditions contains few immune cells other than microglia. Alternatively, there is a rich immune environment within the meninges, consisting of both innate and adaptive immune cells. The BBB is a powerful barrier to immune cells, but can be crossed even when intact. CVOs, particularly the choroid plexus, present a gateway into the CNS. In states of inflammation, immune cells infiltrate the meninges early, which is another potential gateway to the parenchyma.

The function of brain-infiltrating immune cells in states of inflammation is reviewed next. Immune cells infiltrate the brain in a number of neurologic diseases, including Alzheimer's disease (216), stroke (217), Parkinson's disease (218), amyotrophic lateral sclerosis (219), and glioblastoma multiforme (220). Since these

primary neurologic diseases are not the focus of this dissertation, they will not be addressed. Alternatively, MS is driven by brain-infiltrating lymphocytes, and studies on MS were key to gaining understanding on how immune cells infiltrate the brain. Therefore, literature on MS will be discussed first. Next, two conditions (inflammatory liver disease and anxiety) in which mouse model studies identified a role for brain-infiltrating myeloid cells as mediators of sickness behaviors, are discussed.

4.2 Multiple Sclerosis: A potential avenue into understanding how infiltrating leukocytes modulate hypothalamic function

MS is a chronic autoimmune neurodegenerative disease driven by infiltration of myelin-reactive cytotoxic T-cells into the CNS. Motor dysfunction is the most common and most studied symptom, but autonomic dysfunction, cognitive impairment, and metabolic dysfunction are also common, yet do not receive nearly as much attention. MS is driven by an antigen-dependent T-cell response, making it quite distinct from cancer. Nevertheless, much of our understanding of how immune cells from the circulation enter the brain was gained from studies on MS and its associated mouse model, EAE. As such, key studies on MS and EAE relevant to this dissertation will be briefly reviewed, with an emphasis on those that investigate myeloid cells.

In humans, MS lesions are thought to consist mainly of cytotoxic CD8+, myelin reactive T-cells (221). However, other studies show that macrophages constitute the majority of immune cells within MS lesions (222, 223). Until recently, there were no tools to differentiate infiltrating macrophages from microglia, but now it is appreciated that these cells have disparate roles in disease progression (224). While there is altered circulating neutrophil activity during MS (225), granulocytes are not commonly

found in MS lesions (226). Alternatively, in EAE, neutrophils constitute a relatively large majority of infiltrating immune early in disease progression (227) and neutrophil-derived IL-1β is thought to play a key role in disease pathogenesis (228). While these studies presented mainly correlative data, continuous neutrophil depletion can completely prevent clinical symptoms from developing during EAE (229), implicating neutrophils as drivers of EAE. The discrepancies between animal models and human pathology illustrate the challenges in relying on animal modes to study disease.

Studies on MS and EAE provide a wealth of knowledge on how immune cells enter the brain. Despite significant discoveries as to how these cells infiltrate the CNS, their function once within brain is still not well understood. There is general consensus that infiltrating immune cells destroy myelin and kill oligodendrocytes, yet there is still debate as to the role of myeloid cells (230). Furthermore, the key cytotoxic molecules produced by infiltrating immune cells are still not known. While cachexia is not commonly reported in MS, mice with EAE lose significant body mass and develop anorexia (231). Fatigue is also common symptom during MS, implicating the hypothalamus as potentially dysfunctional during MS. Therefore, Chapter 2 will discuss current literature regarding hypothalamic dysfunction, with an emphasis on fatigue and weight dysregulation.

4.3 Lessons from studies on inflammatory liver disease

In contrast with primary CNS inflammatory diseases, few studies investigated infiltrating immune cells in the brain during chronic systemic inflammation. Many focus on sepsis (232-234) (however, it is worth noting that the presence of infiltrating immune

cells in the brain during sepsis is still a topic of debate). There are a few labs that investigated the function of infiltrating myeloid cells in the CNS during diseases with signs and symptoms similar to those seen in cachexia. The excellent work from Dr. Mark Swain's lab at the University of Calgary on inflammatory liver disease is an example. These studies present several important findings on the consequences of myeloid cell infiltration into the brain (235). To study this phenomenon, the Swain lab developed a reproducible mouse model of inflammatory liver disease induced by ligation of the common bile duct (236). These animals develop all of the characteristic features of cholestasis, including increased plasma bilirubin, dark urine, icteric plasma, and increased concentration of liver enzymes in the circulation (179, 237). Kerfoot et al. first reported a significant increase in infiltrated monocytes, defined as CD45^{high}CD11b+Mac1+, in the brains of animals that underwent bile duct ligation (BDL). The identity of these cells as marrow-derived myeloid cells was confirmed in a subsequent BDL experiment that incorporated adoptive transfer of carboxyfluoresceinsuccinimidyl ester (CFSE)-labeled splenocytes. Increased numbers of CFSE-positive cells were found in the brains of BDL mice, which were phenotyped as monocytes. In addition, it was also reported that many of the infiltrating monocytes expressed TNF-α, and that this process was accompanied by microglia activation. Lastly, the authors reported that antibody-mediated blockade of the leukocyte adhesion molecules P-selectin and α4 integrin significantly attenuated monocyte infiltration into the brain in BDL mice (179). In a follow-up study published in the *Journal of Neuroscience*, D'Mello et al. showed that monocytes infiltrated mainly in CVOs (although they did not mention the hypothalamus), the caudate putamen, the

hippocampus, and cortex. In addition, the authors showed that a large percentage of microglia expressed CCL2 in BDL mice, which accompanied an increase in CCR2-expressing brain-infiltrating monocytes. Both CCR2 and CCL2 knockout BDL mice showed attenuated sickness behaviors compared to WT BDL mice (178). It is important to note that in this study, and all subsequent studies from this group, "sickness behaviors" were based solely on social interaction time. In another follow-up study also published in the *Journal of Neuroscience*, D'Mello et al. showed that microglia are activated in BDL mice, and that TNF-α signaling is key for the process. The authors also reported altered neuronal excitability in BDL mice, as indicated by increased seizure threshold. TNF-α was identified as a key inflammatory mediator that was required for monocyte-cerebral endothelium interactions, microglia activation, and sickness behavior. Lastly, P-selectin was also demonstrated as key for sickness behavior in BDL mice (237).

Several limitations to these studies must be considered. First, the phenotyping of the infiltrating immune cells was limited. While the authors focused on monocytes, they did not phenotype any other myeloid cell population (e.g., neutrophils). They did claim that lack of decrease in leukocyte rolling after administration of an anti-Gr-1 ruled out neutrophils. However, a relatively simple flow cytometry experiment could have supported this claim much more robustly. Secondly, none of the interventions used were brain specific. While one paper showed no changes in circulating liver enzymes after P-selectin blockade, implying their intervention did not change liver pathology, the authors did not address how their systemic interventions affected immune cell infiltration in other organs. Nevertheless, these studies provide robust evidence showing that

infiltrating immune cells in the brain are capable of mediating behavior during chronic systemic inflammation.

4.4 Repeated Social Defeat: An illustration of infiltrating leukocyte-driven behavior modulation

Over the last ten years, the labs of Drs. John Sheridan and Jonathan Godbout at Ohio State University have published a series of papers describing how, in a mouse model of anxiety, infiltrating monocytes can modulate behavior. These studies show that during anxiety mice experience significant neuroinflammation, including microgliosis and upregulation of cytokine, chemokine, and leukocyte adhesion molecule transcripts (238). While these phenomena were reported previously in models of depression and anxiety (239), the most novel finding of these papers was that bone marrow-derived monocytes infiltrate the brain after several cycles of repeated social defeat (240). They first described this phenomenon in a paper published in 2013 in *Journal of Neuroscience*, in which they reported that bone-marrow derived monocytes, defined as CD45^{hi}CD11b+, infiltrated several regions of the brain important for memory and activity level, including the prefrontal cortex, hippocampus, paraventricular nucleus of the hypothalamus, and amygdala. To show that monocyte infiltration into the brain contributes to anxiety, the authors utilized CCR2 knockout (CCR2KO) animals. Interestingly, CCR2KO mice had decreased anxiety after RSD compared to WT animals. This was accompanied by a decrease in monocyte infiltration in the brain (241). They then showed that RSD induces myelopoiesis in the spleen, and that splenectomy prevented monocyte infiltration in the brain, as well as anxiety-like behavior after several cycles of RSD (242). In a subsequent study, Wohleb et al. showed, by administering beta blockers to mice

undergoing RSD, that sympathetic signaling was required for splenic monocytes to exit the spleen and infiltrate the brain. Beta blockers also prevented reestablishment of anxiety in RSD-sensitized mice (243). Next, in a pair of descriptive papers, it was shown that the brains of mice experiencing RSD showed microgliosis, increased expression of leukocyte adhesion molecules, increased expression of inflammatory cytokine transcripts, increased expression of chemokine transcripts, and decreased hippocampal neurogenesis (244, 245). Lastly, it was shown that animals that experience RSD also experience memory deficits and HPA axis activation (244, 246)

These studies demonstrate, with extensive evidence, the importance of neuroinflammation for anxiety-like behaviors in mice experiencing RSD. It should be noted that the authors often used the terms "anxiety", "stress", and "depression" interchangeably, as these behaviors are exceedingly difficult to differentiate using murine behavior studies. Furthermore, in their excellent review in *Nature Neuroscience*, Wohleb et al. suggest that depressive/anxiety behaviors share similar features, and perhaps a similar pathogenesis (247). As such, this proposed mechanism of brain infiltrating monocyte-driven "anxiety-like" behavior should be considered generalizable to other models of chronic stress (such as cancer). Indeed, mice experiencing RSD share similar features to those with cachexia, including fatigue and decreased social interaction. Anxiety was recently demonstrated in a mouse model of cancer cachexia (104), but this has not yet been reported in humans. While the Sheridan/Godbout groups did not investigate anorexia in their studies, anorexia often co-occurs with depression (248), anxiety (249), and chronic stress (250). Furthermore, other groups showed that anorexia occurs in different models of chronic social defeat (251). All of

these signs and symptoms are alterations in physiological functions controlled by the hypothalamus, and brain-infiltrating leukocytes should be considered as a unifying mechanism.

5. Summary

Cachexia is a devastating metabolic syndrome consisting of tissue catabolism, anorexia, and fatigue. This syndrome occurs during several diseases, but is most prevalent in cancer. Mechanisms of cachexia remain elusive, but all diseases with associated cachexia are connected by inflammation. Studies on sickness behavior provide a framework for establishing means by which inflammation is translated into anorexia, fatigue, and weight loss. All of the processes dysfunctional in cachexia are controlled to at least some degree by the brain, particularly the hypothalamus. Cancer cachexia is associated with inflammation in the hypothalamus, but cellular sources of inflammation are still not known. This dissertation will describe literature and studies investigating two possible cellular mediators of cancer cachexia: brain-infiltrating leukocytes, and microglia. Molecular mediators key for inflammatory signaling involving these cell types will also be explored.

Chapter 1: The Central Role of Hypothalamic Inflammation in the Acute Illness Response and Cachexia.

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The central role of hypothalamic inflammation in the acute illness response and cachexia

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Abstract

When challenged with a variety of inflammatory threats, multiple systems across the body undergo physiological responses to promote defense and survival. The constellation of fever, anorexia, and fatigue is known as the acute illness response, and represents an adaptive behavioral and physiological reaction to stimuli such as infection. On the other end of the spectrum, cachexia is a deadly and clinically challenging syndrome involving anorexia, fatigue, and muscle wasting. Both of these processes are governed by inflammatory mediators including cytokines, chemokines, and immune cells. Though the effects of cachexia can be partially explained by direct effects of disease processes on wasting tissues, a growing body of evidence shows the central nervous system (CNS) also plays an essential mechanistic role in cachexia. In the context of inflammatory stress, the hypothalamus integrates signals from peripheral systems, which it translates into neuroendocrine perturbations, altered neuronal signaling, and global metabolic derangements. Therefore, this review will discuss how hypothalamic inflammation is an essential driver of both the acute illness response and cachexia, and why this organ is uniquely equipped to generate and maintain chronic inflammation. First, the review will focus on the role of the hypothalamus in acute responses to dietary and infectious stimuli. Next, it will discuss the role of cytokines in driving homeostatic disequilibrium, resulting in muscle wasting, anorexia, and weight loss. Finally, it will address mechanisms and mediators of chronic hypothalamic inflammation, including endothelial cells, chemokines, and peripheral leukocytes.

Key Words: hypothalamus, cachexia, neuroinflammation, cytokines

1. Introduction

Sickness behaviors and their associated metabolic responses are among the most ubiquitous and readily identifiable aspects of acute and chronic illness. In the context of acute threats, the onset of fever, anorexia, lethargy, and catabolism of lean body tissues all evolved as important defenses to promote survival. However, with chronic and ongoing inflammation, these same defenses prove to be a double-edged sword, leading to neurodegeneration, psychiatric conditions, and cachexia. Cachexia is an important predictor of morbidity and mortality in a diverse array of conditions ranging from infectious disease to cancer. Though a large body of work has focused on the direct interaction of cytokines and inflammatory mediators with muscle tissue in the development of cachexia, these direct effects do not account for all aspects of body mass alterations in acute and chronic illness. An increasing body of work suggests the central nervous system (CNS) is a key mechanistic force in the pathogenesis of cachexia by sensing inflammation, integrating information from peripheral organ systems, and evoking downstream changes in body mass and metabolism.

Decades of investigation provide ample evidence that the CNS functions as both a receiver and amplifier of peripheral inflammatory insults, thereby orchestrating a behavioral and metabolic program that leads to wasting and debilitation if prolonged. As a demonstration of neuronal signaling leading to amplified response to inflammation, intracerebroventricular (ICV) injection of pro-inflammatory cytokines potently induces anorexia, lethargy, and catabolism at doses far below the threshold for response with peripheral injection (252-255). Collectively, existing data support a model wherein peripheral inflammatory insults are amplified and modified within the mediobasal

hypothalamus (MBH), creating a paracrine inflammatory milieu that in turn initiates and sustains alterations in the activity of neuronal populations that regulate appetite and metabolism. The attenuated blood brain barrier (BBB) and dynamic regulation of vascular access found in this region is undoubtedly one of the reasons this brain area is highly sensitive to a number of metabolic and inflammatory signaling molecules (256). This review will focus on recent insights in how stimuli of hypothalamic inflammation, including dietary, infectious, and neoplastic sources, are sensed in the CNS and translated peripherally into sickness responses and cachexia.

1.1 Central nervous system control of body mass and energy homeostasis

Within the MBH, there are several neuronal populations in various nuclei responsible for regulation of appetite, body mass, and energy homeostasis. These populations include anorexigenic and orexigenic neuronal subsets, which decrease and increase appetite and food intake respectively. Key anorexigenic populations include pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) expressing neurons (257). POMC is a precursor polypeptide that can be differentially processed for production of αMSH, ACTH, and opioid peptides β-endorphin and μ-encephalin (258). POMC neurons release the anorexic neurotransmitter αMSH, which binds to type 4 melanocortin receptors (MC4R) in a number of downstream sites in the brain to decrease appetite and energy storage. CART is a neuropeptide first discovered as a transcriptional target of cocaine and amphetamine exposure, but was subsequently found to act as an endogenous psychostimulant by increasing locomotor activity and decreasing food intake (259). Conversely, two key orexigenic populations include neurons expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY).

AgRP was first characterized because of a curious connection between pigmentation and metabolic phenotypes, both of which act through melanocortin receptors: mice carrying the dominant *Agouti* allele known as lethal yellow not only demonstrate a yellow pigmentation, but are profoundly obese (260). Further characterization determined that the counter-regulatory neuropeptide AgRP functions as an antagonist of the MCR4 receptor and AgRP neurons directly inhibit POMC neuronal activity, producing increased appetite and energy storage (261, 262). NPY was first discovered as structurally and biologically similar to the intestinal peptide YY (PYY), and is a potent orexigen in the brain and autonomic nervous system (263, 264). Both anorexigenic and orexigenic neuronal circuits are affected both by physiologic cues and by pathophysiologic signals such as cytokines and pathogen-associated molecular patterns (PAMPs) (265). Therefore, the MBH is a key central target of cytokine signaling and provides an important primary neuronal substrate linking inflammation to muscle catabolism, anorexia, and other sickness responses (266-270).

2. The brain-muscle axis: a model for hypothalamic mechanisms of sickness response and cachexia

In states of illness, the body initiates several processes to mobilize energy stores and provide anabolic building blocks for the acute phase response. While important for a healthy immune reaction, this can result in pathologic homeostatic disequilibrium, where the body assumes a state of energy wasting. As a result, tissue breakdown occurs in organs important to activities of daily living. Muscle is an abundant source of amino acids, and often the main target of pathologic tissue breakdown in disease. Selective catabolism of skeletal muscle is one of the hallmarks of cachexia. It is well

known that prolonged systemic inflammation causes skeletal muscle breakdown (271-273). While much of the literature has focused on catabolic pathways within muscle itself, a growing body of evidence indicates the CNS is a key mediator in this process (274-276). In particular, the hypothalamic-pituitary (HPA) axis promotes catabolism of carbohydrates, lipids, and proteins in peripheral tissue in response to cytokines that enter the CNS. As a result, adipose tissue undergoes lipolysis, and proteolysis occurs in skeletal muscle (276). However, skeletal muscle proteolysis occurs selectively over lipolysis in states of neuroinflammation (124).

2.1 Skeletal muscle catabolism is mediated by hypothalamic cytokines

A series of studies demonstrate that through the actions of the prototypical inflammatory cytokine IL-1β, a cascade of signaling is initiated in the hypothalamus, resulting in both local and systemic changes in gene expression, protein synthesis, and neuroendocrine signaling (124, 274, 275, 277). In a rodent model of cancer cachexia, hypothalamic expression of IL-1β mRNA was increased. When IL-1β was administered ICV, animals experienced profound muscle wasting. This effect did not occur when it was administered IP (124), supporting the hypothesis that central, rather than peripheral inflammation is the main instigator of muscle catabolism in this model of cachexia.

While hypothalamic IL-1β alone can induce muscle atrophy (124), other pro-inflammatory cytokines are implicated. However, there is conflicting evidence in regards to their significance and additional research is needed to fully elucidate their mechanisms (277). For example, IL-6 is well established as an important mediator in muscle metabolism (278, 279) but its role within the hypothalamus is not well known.

Although systemic inflammation increases levels of IL-6 in the hypothalamus in concordance with altered energy homeostasis (280), the effect of central IL-6 on skeletal muscle has not been examined. Furthermore, TNFα can directly induce skeletal muscle catabolism (281-283) and produces anorexia when administered centrally (284, 285), yet ICV administration of TNFα in rodents does not induce skeletal muscle thermogenesis (284). However, follow-up studies are lacking, and thus it remains to be discovered whether hypothalamic TNFα, IL-6, or other proinflammatory cytokines are important to skeletal muscle catabolism. As such, this remains an important area of research in the pathophysiology of cachexia.

2.2 Role of neuroendocrine modulation of muscle mass

Following the onset of hypothalamic inflammation, a neuroendocrine signaling cascade is initiated, including marked activation of the HPA axis (256). These neuroendocrine mediators can act directly on skeletal muscle, or act on other organs to amplify the inflammatory signal. It is also likely that CNS mediated activation of the sympathetic nervous system contributes to the disordered muscle metabolism in cachexia, including inhibition of protein synthesis as well as proteolysis within skeletal muscle and myofibril breakdown (124). Endogenous glucocorticoids such as cortisol (or corticosterone in rodents) are important in their ability to modify metabolism of fats, carbohydrates, and proteins, as well as exert control over immune response in times of stress. The chief regulation of glucocorticoid release in humans is at the level of the hypothalamus and pituitary, which produce corticotrophin releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) respectively. CRH, manufactured in the

paraventricular nucleus (PVN) of the hypothalamus, provides the initiating signal of the HPA axis by promoting release of ACTH from corticotroph cells of the anterior pituitary. Direct stimulation by ACTH causes production and release of cortisol from the zona fasiculata of the adrenal cortex (286). Broadly speaking, stress is the key activator of the HPA axis. This system is widely evolutionarily conserved as a method of mounting a defense against stressors including infection and starvation, largely by shifting targets of anabolism and catabolism. While the immune system must undergo tremendous anabolism during stress, with increased production of granulocytes and acute phase proteins, other systems must undergo catabolism in order to provide a supply of biomolecules and energy, particularly in the face of stress-induced anorexia. Skeletal muscle is a major site of protein storage, and serves as a primary target of glucocorticoids. Thus, upon HPA axis activation, skeletal muscle undergoes catabolism to increase plasma levels of free amino acids (124). These amino acids can be used either for production of proteins such as acute phase reactants, or can enter as Krebs cycle intermediates and serve as gluconeogenic substrates in the liver (287). This process is mediated by proteosomal degradation of muscle protein, with E3 ubiquitin ligases, Muscle Atrophy F-box (MAFBx), and Muscle Specific Ring Finger Protein 1 (MuRF1) playing key roles (288, 289).

In the early phases of acute illness, the catabolism of skeletal muscle provides an important energy substrate upon which other defenses can be built. However, when inflammation persists in the context of subacute and chronic disease, this mobilization of protein from skeletal muscle leads to substantial atrophy and functional impairment. The clinical archetype of "matchstick limbs" seen in Cushing's disease, a syndrome

resulting from ACTH-secreting pituitary adenomas, serves as a classic illustration of skeletal muscle derangements evoked by the HPA axis (290). However, Cushing's disease is only one of many conditions known to cause neuroendocrine muscle wasting. Compelling evidence demonstrates that the CNS invokes peripheral muscle wasting via the HPA axis in both cancer and diabetes, with hypothesized roles in numerous other chronic illnesses (277, 291). As such, the CNS not only is tied to the global metabolic dysregulation and behavioral aspects of the sickness response, but it is also able to employ neuroendocrine signaling to invoke an indirect pathway of disease-mediated skeletal muscle atrophy.

3. Mechanisms of hypothalamic inflammation in metabolic derangements: insights from high fat diet and obesity

Consistent with a wide variety of other pathologies, hypothalamic inflammation occurs in both acute and chronic stages. In both instances, the hypothalamus acts in a feed-forward loop to propagate inflammatory responses in the periphery, including changes in behavior and metabolism. To understand how signals arising from the periphery can induce disordered systemic metabolism via a hypothalamic relay, it is illustrative to examine obesity as a systemic inflammatory disorder. In particular, exposure to high fat diet (HFD) is widely studied as a cause of hypothalamic inflammation that in turn leads to significant alterations in body mass regulation and energy homeostasis. Specifically, HFD exposure causes acute inflammation and gliosis in the MBH, which alters metabolic signaling in this part of the brain (292). HFD exposure therefore represents one of several acute inflammatory insults capable of inducing global metabolic derangements via hypothalamic signaling.

Multiple forms of dietary stressors have been investigated for their ability to induce hypothalamic inflammation and downstream alterations in metabolism, including high sucrose diet, high polyunsaturated fatty acid diet, and high saturated fat diet (293, 294). Though all of these diets are capable of producing obesity in experimental models, hypothalamic inflammation and gliosis only consistently ensues in the context of high saturated fat diets. These CNS manifestations substantially precede the onset of overt changes in weight and body mass, occurring within 1-4 days of the onset of a high saturated fat diet (292, 295). A key site of inflammation is the arcuate nucleus, where HFD produces reactive gliosis, increased expression of inflammatory genes IL-1β, IL-6, and TNFα, astrocyte injury, and eventually POMC neuronal injury (170, 292). Further studies indicate that, similarly to peripheral macrophages, microglia of the MBH exhibit an M1-dominant inflammatory response in the presence of saturated fatty acids (170).

To date, studies investigating the mechanisms of HFD-induced hypothalamic inflammatory changes have identified two main pathways: NF-κB signaling and endoplasmic reticulum stress. The hypothalamus demonstrates a different pattern of NF-κB activity compared to peripheral systems, including higher expression of both IKKβ and NF-κB inhibitory protein IκBα, with an overall suppression of NF-κB activity (296). However, with HFD exposure, this dynamic is altered to significantly increase NF-κB activity in the MBH. Forced suppression of NF-κB signaling via MBH-targeted IKKβ knockout results in decreased dietary intake, while MBH-targeted constitutive NF-κB activation results in central insulin and leptin resistance. This same study identified hypothalamic endoplasmic reticulum stress as both an upstream inducer and downstream event of NF-κB signaling (296). Further studies confirm the importance of

NF-κB to hypothalamic energy homeostasis. In both leptin deficient and diet-induced obese (DIO) mice, pharmacologic and genetic inhibition of IKKβ/NF-κB signaling in the arcuate nucleus results in improvements in glucose tolerance and hypothalamic insulin signaling, as well as increasing energy expenditure (297).

The importance of hypothalamic inflammation rests primarily with the fact that the hypothalamus is a central regulator of whole-body metabolism, which produces substantial downstream consequences. Central administration of the saturated fat palmitic acid not only induces a program of hypothalamic inflammation, including increased local expression of cytokines IL-6, IL-1β, and TNFα, but it also leads to decreases in leptin-induced mRNA expression related to gluconeogenesis, glucose transport, and lipogenesis in the liver (298). These peripheral pathological outcomes are abrogated by reduction of hypothalamic inflammation. For example, hypothalamus-specific inhibition of Toll-like receptor 4 (TLR4) and TNFα both result in improved insulin sensitivity in the liver, resulting in decreases in hepatic steatosis and gluconeogenesis (299). However, TLR4-mediated and TNFα-mediated hypothalamic signaling lead to divergent downstream consequences, with only TLR4 signaling inhibition leading to loss of body mass.

Overall, these findings demonstrate that hypothalamic inflammation results as a consequence of high exposure to saturated fat, and through inflammatory changes and recruitment of glial cell populations is able to alter both peripheral metabolism and behavior. As such, HFD models provide a compelling example of how peripherally derived inflammatory stimuli can induce systemically significant pathophysiological changes in the hypothalamus. In contrast to infectious and neoplastic sources of

inflammation, however, HFD eventually produces changes which inhibit anorexigenic POMC and CART neurons, while increasing expression of orexigenic NPY and AgRP. Therefore, hypothalamic inflammation is a potent inducer of peripheral pathophysiological states, the manifestations of which vary substantially with the type and duration of inflammatory stimulus. Through what is in many ways a similar mechanism, other inflammatory insults of the hypothalamus provoke the familiar anorexia, fever, and weight loss known collectively as the acute sickness response.

4. Hypothalamic inflammation and the acute illness response

The most commonly experienced cause of the acute sickness response is logically that which the system directly evolved to combat: the immediate threat of infection. The quintessential traits of acute inflammation and sickness were described as early as Roman antiquity; however, many molecular mechanisms linking inflammation to sickness behavior remain incompletely understood. Even though it is clear that acute inflammation resulting from infection can be deadly, as in sepsis, it is also increasingly clear that acute phase responses are essential to survival. Thus, a significant and growing body of work has focused on understanding the peripheral and central mediators of the response to acute infection, and determining whether each individual step of the process is beneficial or deleterious. The hypothalamus is a vital component of the system responsible for sensing and responding to infectious stimuli, serving as an upstream effector of fever, mobilization of energy stores, and initiation of sickness-associated behaviors. As such, research has focused on two arms of this system: first, how the hypothalamus responds to infectious stimuli on a molecular and

signaling level, and second, how hypothalamic sensing of threats leads to downstream manifestations of sickness.

In its role as a sensor of acute infectious stimuli, the hypothalamus employs a diverse array of danger and pathogen associated molecular pattern (DAMP and PAMP) receptors, as well as being robustly responsive to cytokines and chemokines. Peripheral or central injection of viral or bacterial PAMPs or pro-inflammatory cytokines produces neuronal activation in several brain regions, particularly in nuclei that make up the MBH and its associated vascular structures, collectively known as the median eminence (300-302). Within this region, two key appetite regulating populations of centrally projecting neurons alter their roles as effectors during inflammatory responses: the anorexigenic POMC and CART, and the orexigenicAgRP and NPY.

Much of the current understanding of acute hypothalamic inflammation derives from experiments using lipopolysaccharide (LPS), a PAMP isolated from the outer membrane of gram-negative bacteria. LPS binds to TLR4 to induce canonical NF-κBsignaling, which alters gene transcription to produce a myriad of cytokines, chemokines, and stress response proteins. Both ICV and IP LPS produce acute sickness responses, suggesting it can act through direct interactions with the hypothalamus as well as indirect pathways from the periphery (although with far greater potency after direct CNS administration). Upon exposure to LPS, animals develop sickness-associated anorexia, and orexigenic signaling via NPY decreases at the transcriptional level (303, 304). Similarly, AgRP secretion is decreased following LPS exposure, even though its mRNA levels are increased (268). Conversely, the appetite inhibiting pathways are activated following LPS exposure. With acute LPS stimulation,

POMC neurons are activated, and MC4R and POMC mRNA levels increase (305, 306). Accordingly, both pharmacologic inhibition of MC4R signaling with AgRP, and repression of POMC neuron activation both abrogate the anorexia response following LPS exposure (306-308).

Similar to HFD exposure, the molecular mechanism of LPS-induced hypothalamic inflammation involves NF-κBsignaling via TLR4 and endoplasmic reticulum stress (309). Specifically, TLR4, MyD88, and CD14 are critical to the initiation of sickness behaviors: mice with genetic deletion of any of these proteins demonstrate reduced anorexia in response to IP LPS compared to wild type (303, 310). Fever, anorexia, and hypothalamic inflammation responses to LPS exposure involve the signaling intermediate atypical protein kinase C, whereas hypoactivity and weight loss seem to be mediated by separate pathways (311). Additionally, inducible nitric oxide synthase (iNOS) is induced by LPS exposure in the MBH, where nitric oxide inhibits orexigenic neurons via a STAT dependent mechanism independent of prostaglandin synthesis (312, 313). Obviously, these are important but not exclusive molecular signaling pathways whereby PAMP exposure is translated into behavior, nor is the MBH the only brain region involved. Indeed, it is clear that the brainstem has both redundant and exclusive roles relative to the MBH (for example), but discussion of the entirety of the CNS response to inflammation is beyond the scope of this review.

Though a large body of work has focused exclusively on LPS as an instigator of hypothalamic inflammation of infectious etiologies, viral proteins and nucleic acids are less explored, yet potent inducers of central inflammation. In a study by Jang et al, viral and bacterial components, Tat and LPS respectively, were compared in their effects on

hypothalamic inflammation and resultant sickness behavior. Using IP administration, NF-κB was acutely activated in the MBH in both models, with resultant increased hypothalamic production of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα. In corticotroph AtT20 cells, NF-κB mediated the stimulatory effects of LPS, Tat, and pro-inflammatory cytokines on POMC transcription. Hypothalamic injection of LPS or Tat caused a significant reduction in food intake and body weight, which was prevented by blockade of NF-κB signaling via IKK inhibitory peptide, as well as by blockade of melanocortin signaling via administration of AgRP. Furthermore, specific IKKβ knockout in POMC neurons attenuated LPS- and Tat-induced anorexia. Hypothalamic NF-κB was also activated by ICV leptin (at high pharmacological doses) and mediates leptin-stimulated POMC transcription, suggesting NF-κB is common signaling pathway for all three stimuli (306). In addition to viral proteins, the viral double stranded RNA mimetic poly I:C causes fever, malaise, anorexia, and hypoactivity(314-316). While the fever induced by poly I:C depends partially on IL-1β and IFNα, the mechanism of viral induction of sickness behavior is not yet fully understood – however, this PAMP clearly activates inflammatory signaling and HPA axis activation at the level of the hypothalamus (314, 317, 318).

Importantly, the hypothalamus regulates exposure to inflammatory stimuli differently from peripheral sites of surveillance. While LPS priming decreases the magnitude of cytokine release in the hypothalamus in subsequent LPS exposures, it has the opposite effect in the spleen, where increased levels of IL-1β and IL-1R are induced by repeated exposures (319). Furthermore, IP LPS time-dependently increases STAT3 phosphorylation in both the hypothalamus and liver, but the hypothalamus is

comparatively more acute and transient than the liver in this signaling event (320). Most intriguing of all is the recent discovery that microglia have a distinct ontogeny from the peripheral immune system, which may explain why it differs in its responses to inflammatory events (321). Despite the long-held assumption that microglia originate in bone marrow, akin to other monocytes, fate mapping demonstrates that microglia are seeded from the embryonic yolk sac and remain as a self-sustaining population throughout life. While other tissue macrophages arise from this same embryonic event, the key difference is that only the peripheral tissues continue to recruit from hematopoietic stem cells of the bone marrow throughout life (322). Microglia may recruit from the peripheral monocyte pool during times of profound pathology, such as major insults to the BBB, but they remain an isolated and self-renewing population throughout most physiological states. This discovery is important, as the distinct lineage of microglia suggests they are likely to have distinct molecular signaling pathways to detect and propagate inflammation. These combined data suggest acute hypothalamic inflammation is a process robustly induced by a variety of stimuli, proceeds through distinct signaling modalities compared to peripheral tissues, and requires a combination of signal initiation and signal propagation events. A variety of signaling mediators and anatomical considerations make hypothalamic inflammation a particularly unique and a targetable niche of acute sickness responses and cachexia alike.

4.1 The role of cytokines in acute hypothalamic inflammation and cachexia

While cytokines are key signaling mediators in normal immune responses, high levels are damaging and can lead to sustained inflammation with many detrimental

effects. In cancer, higher levels of cytokines correlate with poorer outcomes (72). Furthermore, elevated circulating levels of TNFα and IL-6 in cardiac cachexia are the strongest predictor for pathological weight loss (323, 324).While cytokines have significant effector functions in various organs including muscle (325), it is their action in the CNS, specifically the hypothalamus, that is the primary driver of the behavioral features of cachexia (120). In spite of the presence of the mostly impermeable BBB, there are several ways cytokines can enter the brain from the circulation. First, since circumventricular organs lack a BBB, most cytokines can enter the hypothalamus by volume diffusion (326). In addition, some cytokines, including IL-6 (327), TNFα (328), and IL-1β (329), can cross through various transporter systems. Lastly, circumventricular organs and the choroid plexus contain macrophage-like cells that express TLRs, allowing cytokines to exert their functions without entering the brain parenchyma (330).

Dozens of different cytokines have been implicated in cachexia. However, in the hypothalamus, the most robust data supports roles for TNFα and IL-1β in cachexia and sickness behavior (331, 332).

4.1.1 TNFα

Although TNFα has direct effects on various target tissues (331, 333, 334), its most potent actions in regard to cachexia occur in the hypothalamus. Within the hypothalamus, the actions of TNFα alone lead to anorexia, thermogenesis, and increased respiratory rate (284, 335, 336). Injection of TNFα into the lateral hypothalamus causes decreased firing rate of neurons in that nucleus (337). In rodents,

ICV injection of TNFα leads to decreased food intake and increased respiratory quotient (336). These symptoms correlate with increased expression of mediators of the JAK/STAT signaling pathway within the hypothalamus, indicating this signaling mechanism is an important mediator in sickness behavior. Furthermore, ICV injection of TNFα leads to reduced food intake and weight loss. In addition to the JAK/STAT signaling pathway, it also was determined that TNFα signaling within the hypothalamus leads to increased thermogenesis in brown adipose tissue via β-adrenergic signaling (284). Further studies are necessary to determine the specificity of different TNFα signaling pathways within the hypothalamus in cachexia.

4.1.2 IL-1β

IL-1β is established as one of the primary molecules in neuroimmune signaling. While IL-1β can act on numerous different types of cultured cells found within the brain, functional IL-1 receptors are primarily localized to endothelial cells and certain neuronal populations, including those within the ARC (338). It is well documented that systemic inflammation increases IL-1β activity in the hypothalamus (124, 339, 340). IP injection of LPS induced increased IL-1β expression in endothelial cells of the PVN (339). Furthermore, it is an important cytokine in the hypothalamic relay leading to skeletal muscle catabolism (124). In rodents, chronic ICV administration of IL-1β induces skeletal muscle wasting (124). In the same study, development of carcinoma-induced cachexia was associated with increased hypothalamic IL-1β expression. In addition, knockout of endothelial IL-1 receptor attenuated expression of IL-1β, TNF-α, and IL-6 mRNA in the brain in a mouse model of chronic stress (341).

5. From acute to chronic: how hypothalamic inflammation contributes to transition from sickness to cachexia

5.1 Pathological transition from acute to chronic inflammation

Although IL-1β and TNFα potently induce acute anorexia, animals rapidly desensitize to continuous ICV administration (342-345). This tachyphylaxis indicates these cytokines are not sufficient to produce sustained catabolism, suggesting other mediators are necessary to maintain and amplify the inflammatory signal. While the mechanism of chronic hypothalamic inflammation in cachexia is not fully understood, several candidates have been identified. The cytokine Leukemia Inhibitory Factor (LIF) is expressed in the ARC, and can induce anorexia in inflammatory disease (253, 346, 347). In contrast to IL-1β and TNFα, chronic administration of LIF does not induce tachyphylaxis(348-350). However, the neuroinflammatory actions of LIF appear to be overlapping with IL-1β, implicating it is a secondary amplifier of inflammatory response, rather than the sole mediator of chronic hypothalamic inflammation (351).

While most studies focus on the actions of neurons within the hypothalamus in producing sickness response, an emerging body of evidence suggests non-neuronal cells play an important role in the transition from acute to chronic inflammation in the CNS in cachexia. Specifically, cytokines initiate a cascade involving endothelial activation, followed by increased expression of cell adhesion molecules, secretion of chemokines, and recruitment of peripheral leukocytes (Figure 1). While the role of these mediators in the hypothalamus is not well known, they represent an important area for future research in the field of cachexia.
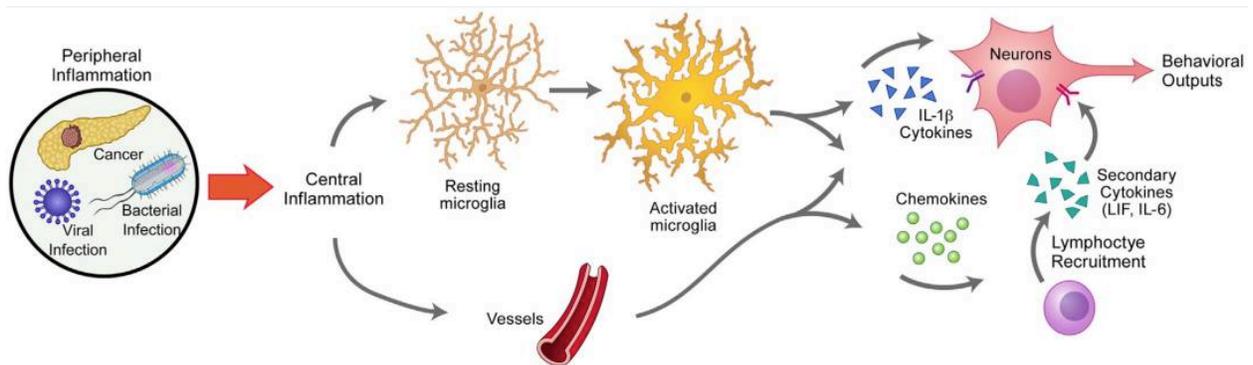
Figure 1. Initiation, amplification, and perpetuation of hypothalamic inflammation.
Repeated CNS exposure to primary inflammatory cytokines results in tachyphylaxis; therefore, additional pathophysiological steps must be involved to maintain chronic inflammation in cachexia. Upon entering the CNS, cytokines bind to PAMP receptors and activate numerous types of cells, including glia and hypothalamic vascular endothelial cells. Microglia are recruited and activated by PAMPs, which results in increased release of cytokines and chemokines. Endothelial cell activation in vasculature results in secretion of IL-1β and additional cytokines, chemokine secretion, and expression of cell adhesion molecules. Chemokines and adhesion molecules in turn recruit leukocytes from the periphery. These cells secrete secondary cytokines, such as IL-6 and LIF. These pathways ultimately converge on neurons to elicit neuroendocrine, metabolic, and behavioral changes

5.2 A reactive endothelium and leukocyte recruitment: new frontiers in neuroinflammatory mechanisms of cachexia

When crossing from the periphery to the CNS, cytokines and other inflammatory molecules first encounter endothelial cells, which express high levels of cytokine

receptors. This interaction leads to secretion of additional cytokines, expression of selectins, production of chemokines, and peripheral lymphocyte recruitment. While the role of these mediators has not been studied extensively in the hypothalamus specifically, there is evidence to suggest they play a key role in the sequelae of cachexia (discussed further, below). Further studies are necessary to determine possible variations within the hypothalamus. Furthermore, these mediators are not yet targeted therapeutically, presenting an unexplored opportunity for therapeutic intervention.

5.2.1 An activated endothelium

Upon exposure to inflammatory products, vascular endothelial cells enhance their primary role in the circulatory system by activating pathways designed to combat pathogens. Endothelial cells express TLRs that serve as "on" switches to an active state, leading to several phenotypic changes. Upon activation, inter-epithelial junctions are downregulated, allowing for immune cells to access tissue and fight pathogens. Furthermore, endothelial cells can produce molecules such as metalloproteases and additional cytokines that are directly toxic to pathogens, as well as adhesion molecules to promote leukocyte migration and extravasation. In the CNS, neuroinflammation activates endothelial cells and other cells associated with the BBB. If the neuroinflammation persists, this can cause BBB breakdown, which is associated with several pathologies such as Alzheimer's disease (352), Parkinson's disease (353), vascular dementia (354), stroke (355), and multiple sclerosis (356).

5.2.1.1 Cytokines and the hypothalamic endothelium

All of the major cytokines involved in cachexia play a role in activating endothelial cells. It is well known that increased expression of cytokines selectively occurs within endothelial cells of circumventricular organs of the hypothalamus during systemic inflammation (330, 339, 340). For example, injection of LPS leads to increased expression of IL-1β in endothelial cells of the hypothalamus (340). This induces the production of numerous cytotoxic molecules, including cytokines, adhesion molecules, matrix metalloproteinases, and coagulation factors (357). In addition, IL-1β activates cyclooxygenase in cerebral endothelium, leading to production of the pyrogenic arachidonic acid metabolite prostaglandin E2 (358). Furthermore, upon exposure to LPS, IL-1β is a key mediator to microglial production of the vasodilator and neuromodulator nitric oxide, via increased biosynthesis of iNOS(359).

Additional cytokines play important roles in endothelial activation as well. In cerebral microvessel endothelial cells, TNFα exposure induces Rho activation and myosin light chain phosphorylation, leading to a gradual increase in permeability and loss of endothelial junctions (360). IL-6 is produced locally by brain endothelium upon LPS injection (361) and can be further induced by IL-1β (362). Lastly, although it does not cross the BBB (363), TGF-β accumulates in cerebral endothelium (364) and is reported to increase BBB permeability during inflammation (365).

While endothelial activation in response to cytokines is not exclusive to the hypothalamus, the circumventricular structures - including the MBH - make this organ uniquely equipped to sense, amplify, and respond to inflammatory molecules. First, the MBH is a highly vascularized structure lacking a BBB, containing mainly fenestrated

capillaries, which provides circulating materials direct access to the parenchyma. Second, due to its adjacency to the third ventricle, the MBH is in direct contact with the CSF, furthering its exposure to circulating solutes within the CNS. Third, within the ependymal lining of the third ventricle are specialized cells called tanycytes. These cells are found exclusively within the ventricular lining of the hypothalamus, and extend processes deep into the parenchyma (366). Although relatively little is known about tanycytes, evidence suggests they are heavily involved in energy homeostasis and hypothalamic neuroendocrine signaling (367). However, the role of these cells in cachexia has not been investigated. Lastly, as described previously, the hypothalamus is the feeding center of the CNS, and many of these areas, including the PVN and ARC, express receptors for these cytokines (338, 368-370).

Extensive vasculature and ample access to cytokine exposure make the hypothalamus an ideal location for signal amplification via endothelial signaling. Furthermore, in response to cytokines, cell adhesion molecules are upregulated and chemokines are secreted, subsequently leading to peripheral immune cell infiltration. All of these factors make the hypothalamus prone to perpetuation of inflammation, which is critical for development of cachexia.

5.2.1.2 Cell Adhesion Molecules

Endothelial adhesion molecules function to tether circulating leukocytes to vascular endothelium, as well as facilitate rolling and migration into tissue. There are several different adhesion molecules expressed on leukocytes and endothelial cells, which fall into two broad categories: selectins, which are expressed on endothelial cells

(with the exception of L-selectin) and integrins, which are expressed on leukocytes (371).

Cellular adhesion molecules have very low levels of basal expression and are only upregulated in inflammatory conditions (372). While expression of these molecules is necessary for an appropriate immune response, they can be markers of pathologic inflammation. Increased expression of adhesion molecules is reported in numerous systemic inflammatory diseases, such as atherosclerosis (373), heart failure (374) inflammatory bowel disease (375), allergy (376), renal disease (377), COPD (378), and cancer (379). While the role of adhesion molecules in local immune responses has been studied extensively in nearly every condition that causes cachexia, their role in the pathophysiology of sickness behavior and cachexia itself is less well known. In patients with cancer cachexia, P-selectin polymorphisms are predictive of increased muscle wasting (380). In an accompanying rodent model of cachexia, the P-selectin gene was a top early-induced gene (380). Furthermore, a follow-up study found P-selectin polymorphisms were associated with increased risk of developing cachexia in pancreatic cancer patients (381). These studies assessed the role of P-selectin in skeletal muscle, rather than the CNS. No studies have investigated the expression and function of P-selectin or other adhesion molecules within the hypothalamus in cachexia. However, these adhesion molecules are highly upregulated in other portions of the brain during acute and chronic inflammation (178, 179, 382). Furthermore, in rodent models of liver inflammation, cellular adhesion molecules within the CNS are implicated as important mediators of sickness behavior (178, 179, 383). In liver disease, there are high levels of cytokines and endotoxins, which result in increased expression of cellular

adhesion molecules, chemokines, and subsequent leukocyte recruitment. Kerfoot et al. reported that in mice with cholestatic inflammatory liver disease, there were increased levels of endothelial adhesion molecule VCAM-1. This correlated with increased levels of monocytes within brain parenchyma. Furthermore, blockade of leukocyte trafficking molecules α-4 integrin and P-selectin abolished this effect (179). In a follow-up study administration of the same blocking antibody combination resulted in a decrease in sickness behavior, quantified by social interaction, in mice with inflammatory liver disease (178).

These results, along with substantial evidence indicating adhesion molecules play a prominent role in other neuroinflammtory diseases such as multiple sclerosis (384), Alzheimer's disease (385), and stroke (386), make it reasonable to suspect these molecules are important in the pathophysiology of cachexia. Furthermore, viral models of cachexia suggest leukocyte recruitment into the CNS, which is mediated by chemokines and adhesion molecules, also has an important role in sickness behavior (387, 388) (see Section 5.2.3).

5.2.2 Chemokines

Chemokines are small proteins that attract and activate immune cells. They are involved in virtually all pathologies with an inflammatory component. While their role in the immune response is well known, it was only recently discovered that they are prominent mediators of CNS response to stress. In the CNS, chemokine receptors are upregulated on astrocytes (389), microglia (390), and neurons (391) during neuroinflammation. In states of stress, hypothalamic chemokines play an important role

in the pathophysiology of cachexia (392). ICV administration of numerous chemokines into the rat brain, including IL-8/CXCL8, IP-10/CXCL10, CCL2 and RANTES/CCL5, decrease short-term food intake (393). During states of stress, these chemokines are expressed mainly in circumventricular organs (392). For example, upregulation of IL-8/CXCL8, the major chemokine in neutrophil chemotaxis, in the PVN is associated with fetal stress during birth (394) and hyperthermia after LPS injection (395). Furthermore, CXCL8 expression in the PVN is also implicated in signaling of stress hormones, such as ACTH and CRH (396).

Various chemokines cause a leukocyte response in the CNS via migration from the periphery into the brain parenchyma. While the role of these molecules in the hypothalamus in cachexia is not yet known, previous studies show they are important in endothelial activation and maintenance of neuroinflammation. For example, Wu et al. showed that knocking out CCR2 in mice prevented endothelial activation and leukocyte recruitment into the CNS after ICV LPS injection (397). Similarly, when CCR2 is knocked out of microglia, monocyte infiltration into the CNS is decreased during systemic inflammation. This subsequently results in decreased sickness behavior, at least as measured by diminished social interaction (178).

Furthermore, CXCL10 is associated with T-cell responses, and regulates the migration of T-cells into the brain parenchyma in response to various neuroinflammatory states (387, 398). It is induced by IFN-Y (388) and in the CNS is expressed almost exclusively in astrocytes (388, 399). While the role of CXCL10 in the hypothalamus has not been studied, mice infected IP with the parasite *Trypanosoma brucei* showed increased CXCL10 expression in the hypothalamus (400). Furthermore, mice with

global CXCL10 deletion show decreased mortality in lymphocytic choriomeningitis virus (LCMV) infection, a viral model of cachexia (see Section 5.2.3) (387, 388). However, these knockout studies did not assess hypothalamic infiltration or sickness behavior. Future studies should investigate whether CCR2 or other chemokine knockouts can attenuate additional inflammation or alleviate cachexia symptoms.

High levels of chemokines within the brain, along with increased expression of adhesion molecules on endothelial cells suggest a mechanism of recruiting peripheral cells into the CNS during neuroinflammation and a potential role for these cells in cachexia. While the role of peripheral leukocytes has not been studied in most forms of cachexia, for over 20 years immunologists have been studying a murine model of viral CNS infection that induces profound wasting. This wasting is mediated almost entirely by infiltration of peripheral lymphocytes into the CNS. As described in the following section, this model presents a powerful means for investigating the role of leukocytes in viral cachexia, and a framework for studying these cells in other causes of sickness behavior.

5.3.1 Leukocyte recruitment

While leukocyte trafficking into the CNS is evident in several neurological diseases that result in cachexia, their role in the pathophysiology of hypothalamus mediated sickness behavior is unknown. However, studies of LCMV infection demonstrate that peripheral leukocytes enter the CNS and play an important role in maintaining cachexia (401-403). T-cells release a number of cytokines capable of producing an illness response when injected centrally, including IL-6 and LIF (404, 405).

In animals inoculated ICV with LCMV, anorexia and lethargy are maintained by MHC II restricted CD4+ cells (401). Mice treated with anti-CD8 antibodies or HLA Class I knockout then infected with LCMV experienced nonlethal chronic wasting, losing approximately 25% body weight over the course of 32 days before recovering (401).

While the LCMV model is used extensively from the immunology standpoint to study persistent infections, it has rarely been used to study cachexia (403, 406). Kampershroer and Quinn infected CD8+ T-cell deficient mice with LCMV via ICV inoculation and found that wasting was dependent on IFN-γ and IL-1, with IL-6 contributing to symptoms (406). However, in contrast to previous cachexia literature, wasting was not dependent on TNFα. In a recent study, Stamm et al. injected WT mice with LCMV virus IV at different doses and sought to identify dose effects and further characterize mediators of wasting. The authors found a positive correlation between weight loss and viral dose, but were unable to attenuate symptoms with IFN-γ or IL-1 blockade (403). Nevertheless, these studies show the LCMV model is a potent inducer of cachexia and these intriguing results suggest it can be a means of making new discoveries in the mechanisms and treatment of cachexia.

Future studies are needed to determine whether lymphocyte recruitment in LCMV models is a byproduct of elevated numbers of lymphocytes recruited systemically to fight infection, or a mediator of hypothalamic inflammation and an integral part of the mechanism of cachexia. In addition to LCMV, other mouse models of sickness behavior demonstrate that systemic inflammation induces leukocyte recruitment in the CNS. In models of inflammatory liver disease, monocytes were found in various regions of the brain and produced TNFα, amplifying the neuroinflammatory response (178, 179).

Blocking entry of these molecules to the CNS attenuated sickness behavior. In contrast to LCMV studies, the leukocyte population entering the CNS was almost exclusively monocytes. Unfortunately, the hypothalamus was not assessed as a potential location for leukocyte recruitment. Future studies are needed to investigate whether monocytes play a role in the hypothalamus in inflammatory liver disease.

Lastly, in a rodent model of chronic stress, monocytes were reported to infiltrate the CNS, including the PVN (341, 407). Future studies will be necessary to assess whether leukocyte recruitment is specific to the hypothalamus and determine the characteristics and effector functions of these cells.

Recent studies demonstrate that peripheral leukocytes enter the brain in cachexia and play a role in the neuroinflammatory response that causes sickness behavior. In addition to the studies mentioned above, studies in cancer immunology have shown that leukocytes play an important role in modulating inflammatory immune response in the brain (408). However, the specificity and characteristics of these cells have yet to be fully elucidated. Further studies are needed to determine if these cells enter the hypothalamus, and the role of their effector functions.

6. Concluding Remarks

Whether in the context of acute or chronic inflammatory insults, a growing body of evidence implicates the hypothalamus as a driving force in the pathophysiology of the acute sickness response and cachexia. The hypothalamus orchestrates orexigenic and anorexigenic drives in both physiological and pathophysiological states. A variety of dietary stressors, infectious stimuli, and chronic illnesses are able to alter the behavior

of these cell populations. Anatomical considerations such as the attenuated BBB of the median eminence allow direct sensing of DAMPs and PAMPS, as well as indirect sensing of threats via peripheral immune responses giving rise to cytokines. Hypothalamic exposure to any of numerous inflammatory stimuli triggers an acute sickness response, caused by IL-1β and TNF-α, leading to fever, lethargy, anorexia, and weight loss. These molecules act acutely by binding to receptors on circumventricular neuronal populations, such as POMC and AgRP, triggering a feed-forward loop involving skeletal muscle catabolism and lipolysis. While cytokines are important mediators of acute sickness response, their effects are rapidly attenuated and undergo tachyphylaxis over time, implicating additional mediators in chronic hypothalamic inflammation. The key paracrine and autocrine signaling pathways local to the hypothalamic neurons and glia remain yet to be fully elucidated, but the recent discovery of the separate ontogeny of microglia from peripheral immune cells provides an exciting opportunity for understanding how inflammation differs in the CNS. Furthermore, cytokines can also act on endothelial cells within the rich capillary supply of the hypothalamus, leading to further production of cytokines, increased expression of cell adhesion molecules such as selectins and integrins, and secretion of chemokines. Subsequently, peripheral leukocytes are recruited into the CNS, where they secrete cytokines and amplify neuroinflammation. While LCMV and chronic liver inflammation rodent models provide insight on the role of peripheral leukocytes in cachexia, no studies have addressed their function within the hypothalamus. Further studies and characterization are needed to determine whether these cells enter the MBH or adjacent structures in states of inflammation. Even though many unknowns persist, one

thing remains clear: the hypothalamus is crucial in both the acute sickness response and cachexia, and an improved understanding of its role in these processes could prove essential in uncovering the elusive therapeutic solution for these conditions.

Chapter 2: Hypothalamic Dysfunction in Multiple Sclerosis: Implications for Fatigue and Weight Dysregulation

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Title: "Hypothalamic Dysfunction and Multiple Sclerosis: Implications for Fatigue and Weight Dysregulation"

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Abstract

Signs and symptoms of multiple sclerosis are usually attributed to demyelinating lesions in the spinal cord or cerebral cortex. The hypothalamus is a region that is often overlooked yet controls many important homeostatic functions, including those that are perturbed in multiple sclerosis. In this review we discuss how hypothalamic dysfunction may contribute to signs and symptoms in people with multiple sclerosis. While dysfunction of the hypothalamic-pituitary-adrenal axis is common in multiple sclerosis, the effects and mechanisms of this dysfunction are not well understood. We discuss three hypothalamic mechanisms of fatigue in multiple sclerosis: 1) general hypothalamic-pituitary-adrenal axis hyperactivity, 2) disordered orexin neurotransmission 3) abnormal cortisol secretion. We then review potential mechanisms of weight dysregulation caused by hypothalamic dysfunction. Lastly, we propose future studies and therapeutics to better understand and treat hypothalamic dysfunction in multiple sclerosis. Hypothalamic dysfunction appears to be common in multiple sclerosis, yet current studies are underpowered and contradictory. Future studies should contain larger sample sizes and standardize hormone and neuropeptide measurements.

Keywords: Multiple sclerosis**,** hypothalamus, fatigue, weight dysregulation, neuroendocrinology

1. Introduction

Multiple Sclerosis (MS) is an autoimmune demyelinating disease that affects over 2.3 million individuals worldwide (409). MS pathogenesis is driven primarily by autoreactive immune cells attacking myelin and axons of central nervous system (CNS) neurons, leading to destructive lesions in the spinal cord and brain. These lesions lead to motor, sensory and cognitive impairment, and autonomic dysfunction (410-412). Additionally, there are other signs and symptoms that are not typically linked to CNS lesions and yet can cause significant disability. These include fatigue and metabolic dysfunction. The etiologies of these signs and symptoms are undoubtedly multifactorial. However, the hypothalamus is known to regulate a number of homeostatic systems, including sleep, motivated behaviors, appetite, and basal metabolic rate. Hypothalamic dysfunction is common in chronic inflammatory diseases similar to MS (413, 414), yet this region of the CNS is often overlooked when considering underlying etiologies and treatment of signs and symptoms of MS.

The hypothalamus is anatomically configured to allow neurons and other cells to detect circulating factors, hormones, and metabolites in order to adjust homeostatic function in response to stressors and physiologic perturbations. It is located adjacent to the third ventricle in close contact with circumventricular structures and contains a rich supply of fenestrated capillaries with an attenuated blood brain barrier (BBB). Neurons in this region of the brain are highly responsive to immune mediators (415) and cytokines directly modulate the activity of multiple hypothalamic neurons (267, 416, 417). While this response is important to maintaining homeostasis, chronic hypothalamic inflammation is maladaptive, and is associated with a variety of systemic pathologies

including muscle wasting, disordered sleep, insulin resistance, and fatigue (121). Hypothalamic dysfunction is particularly prevalent in inflammatory diseases in which patients experience increased levels of circulating cytokines and other pro-inflammatory molecules, such as cancer (418), rheumatoid arthritis (419), congestive heart failure (420), and cirrhosis (421). In the setting of obesity, it is hypothesized that increased circulating cytokines leads to hypothalamic inflammation, which causes aberrant activity of weight regulatory neurons in this region, contributing to the pathogenesis of this disease (422).

Hypothalamic-pituitary-adrenal (HPA) axis dysfunction is common in MS (423-426), yet its underlying etiology is poorly understood. Possibilities include damage to the hypothalamus or a secondary effect of a global stress response to the disease. The few studies exploring the prevalence of hypothalamic lesions in MS report a high prevalence of lesions in this brain region. For example, Huitinga et al. investigated hypothalamic lesions in MS patients and reported that 16 of 17 had lesions in this region (427). Increased HPA axis activity, quantified by cerebrospinal fluid (CSF) cortisol levels and number of corticotrophin releasing hormone (CRH)-producing neurons in the paraventricular nucleus of the hypothalamus, is also associated with increased disease severity in MS (428).

There are currently no guidelines for recognizing or managing hypothalamic dysfunction in MS. Furthermore, reports on causes and mechanisms of resultant signs and symptoms are conflicting. There are no reviews summarizing literature on the effects, mechanism, and potential therapies for hypothalamic involvement in MS. In this review we summarize the current literature on hypothalamic dysfunction in MS in the

context of fatigue and weight dysregulation. We then propose treatments and recommend future studies.

2. Fatigue

2.1 Fatigue in MS

Fatigue affects 50-90% of people with MS (429, 430). For many people with MS, fatigue is reported as the most debilitating symptom, often surpassing both motor impairment and autonomic dysfunction (431). Despite this serious clinical concern, this complex syndrome is poorly defined, with most diagnostic criteria relying on subjective self-reporting by patients (432). Mechanisms of fatigue are also poorly understood, and dissecting fatigue from sleep disturbances is difficult. Up to 50% of people with MS suffer from sleep disorders (433), which often occurs concurrently with fatigue (434). Furthermore, numerous pharmacologic agents commonly used in MS cause fatigue (435-437). These confounding factors make studying fatigue in humans and animal models challenging. Future studies are needed to fully understand the relationship between insomnia and fatigue in MS. However, there is general consensus that fatigue in MS originates in the CNS (438). Within the CNS, the actions of neurons that project to and from the hypothalamus control wakefulness and activity levels. As such, many studies investigated the role of hypothalamic dysfunction in disorders of wakefulness in MS. There are differing theories of the key substrate within the hypothalamus responsible for fatigue. This section will describe three different theories: 1) overall HPA axis hyperactivity 2) disordered orexin neurotransmission and 3) abnormal cortisol secretion.

2.1.1 HPA Axis Hyperactivity

As mentioned earlier, several studies demonstrate that many people with MS have increased HPA axis activity. Previous studies investigating HPA axis and fatigue in other chronic diseases are conflicting. Some studies have found fatigue and general HPA axis hyperactivity to be correlated in chronic fatigue syndrome (CFS) (439), Alzheimer's disease (440), major depression (441), cancer (442), and rheumatoid arthritis (443). However, other studies have found HPA axis hypoactivity in people with fatigue due to cancer (444), fibromyalgia (445) and CFS (446). These conflicting results may occur due to the difficulty of quantifying HPA axis activity. Many studies (including several of those listed above) use serum or urinary cortisol as a measure of HPA axis activity, which are often unreliable and difficult to standardize. However, it is generally accepted that in states of chronic disease and stress the HPA axis is highly activated (447), which can alter levels of wakefulness (448).

In MS, few studies have investigated the relationship between HPA axis activity and fatigue. Gottschalk et al. (449) used the dexamethasone-CRH test to quantify HPA axis activity in 31 individuals with relapsing-remitting MS (RRMS). Fatigue was measured through three questionnaires: the Fatigue Severity Scale, the Modified Fatigue Impact Scale, and the Visual Analog Scale. The authors found that individuals with fatigue had significantly elevated adrenocorticotropin (ACTH) levels after CRH administration compared to those without fatigue. It is important to note that individuals without MS were not included in this study. Alternatively, Heesen et al. (450) was unable to find a relationship between HPA axis activity and fatigue in 30 MS patients. A

possible reason for this discrepancy is that in this study both chronic progressive phase and RRMS patients were included, while in Gottschalk et al. only RRMS patients were included. Further studies with larger, more homogenous patient samples are needed to better understand the role of HPA axis hyperactivity in fatigue in MS.

2.1.2 Orexin

A neurotransmitter that is of particular interest in both fatigue and sleep dysfunction is orexin. Orexin is a neuropeptide synthesized by neurons in a small number of nuclei within the hypothalamus that project widely to brain areas involved in arousal and motivated behaviors. It activates monoaminergic and cholinergic wake-active neurons in the hypothalamus and brainstem during the daytime (451). Orexin neurons located in the perifornical and dorsomedial hypothalamic areas project to numerous areas in the brain, including the locus ceruleus, septal nuclei, stria terminalis, and parbrachial nuclei (452). The activity of these neurons is critical for maintenance of long, consolidated awake periods (453). Deficiency of orexin signaling in humans leads to narcolepsy, a sleep disorder characterized by inability to maintain a vigilant state and pathologic intrusion of rapid eye movement sleep during awake periods (453). Furthermore, loss of orexinergic neurons or dysfunction in the orexinergic system has been observed in other CNS diseases such as Alzheimer's disease (454), and in animal models of Parkinson's disease (455) and stroke (456). In addition, neuroinflammation reduces hypothalamic orexin neuron activity, which leads to fatigue (457).

Orexin dysfunction is hypothesized as one cause of fatigue in MS, but studies investigating this relationship are conflicting. There are several case reports of MS

patients with hypothalamic lesions with low CSF orexin levels and accompanying hypersomnia or fatigue (458-460). For example, Kato et al. (458) showed that the CSF concentration of orexin in a person with MS with bilateral hypothalamic lesions was much lower than normal. Oka et al. (459) reported similar findings in a 22 year-old female with MS who presented with hypersomnia and was found to have low CSF orexin and a hypothalamic lesion.

Cohort studies of MS patients, as well as animal studies, also showed altered levels of orexin associated with fatigue. The results of these studies are conflicting and there is no consensus on whether increased or decreased levels of orexin are associated with fatigue in MS. Küçükali et al. (461) reported that serum orexin levels were reduced in experimental autoimmune encephalitis. In direct contrast, Papuc et al. found a *positive* correlation between CSF orexin levels and fatigue severity score (FSS) in 38 people with MS. This correlation was even stronger in the MS subgroup that suffered from fatigue (462). However, the authors did not analyze this correlation in the 15 age-matched non-diseased control subjects included. These results are surprising, since fatigue is associated with decreased wakefulness, yet increased orexin levels are associated with increased wakefulness. Therefore, the expected result would be a *negative* correlation between CSF orexin levels and FSS. The authors suggested their findings may be a result of a compensatory mechanism.

Alternatively, Constantinescu et al. (463) investigated the correlation between CSF orexin levels and FSS in 34 patients with MS, 24 patients with other inflammatory neurological diseases (neurosarcoidosis, clinically isolated syndromes, encephalitis or meningitis, and inflammatory demyelinating polyradiculopathies), and 42

patients with non-inflammatory neurological diseases (idiopathic intracranial hypertension, cerebrovascular disease, primary headache syndromes, and axonal neuropathies) and reported no correlation between CSF orexin levels and FSS in any of the three groups.

It is important to note that in both Küçükali et al. and Constantinescu et al., levels of orexin did not significantly differ between MS and non-MS groups (although in both studies CSF orexin levels were decreased in MS individuals compared to individuals without MS), different MS subgroups, or individuals with and without fatigue.

These conflicting and surprising results suggest additional studies are needed to better understand disordered orexin neurotransmission in MS and its role in fatigue. One potential cause of these conflicting results is the inherent challenge in obtaining a sufficient number of accurate orexin measurements. Since orexin most likely does not readily cross the BBB, it must be measured from the CSF (464). As such, recruiting a sufficient number of non-diseased individuals for CSF samples is always challenging. Furthermore, the two studies used different orexin detection assays, which are not cross-validated. In addition, although samples were taken during the day in both studies, orexin levels vary throughout the day (465), and even seasonally (466). Finally, developing a better understanding of whether or not the diurnal variation in orexin levels is disrupted in MS patients is essential. Diurnal variation in orexin activity is critical to avoid fatigue. Low levels at night promote consolidated sleep, while high levels during the day promote activity (467). This is important for developing a therapeutic target of orexin. Orexin receptor antagonist use at night and/or agonist use during the day should be considered. This will be discussed further in Section 5.

2.1.3 Cortisol

As discussed in Section 2.1.1, perturbations in HPA axis activity occur in many chronic diseases. One of the key hormones of the HPA axis implicated in fatigue and wakefulness is cortisol. Cortisol is a glucocorticoid produced in the zona fasciculata of the adrenal gland. Its release is stimulated by the pituitary peptide ACTH, which in turn is stimulated by CRH produced in the hypothalamus and secreted into the portal vasculature. Cortisol is important in several homeostatic functions, including metabolism (468), immune function (469), and wakefulness (470). It is released in response to a variety of stressors (471), and perturbed cortisol secretion is predictive of poorer outcomes in both neurologic and non-neurologic diseases (472-474). Cortisol contributes to wakefulness mainly through the cortisol awakening response (CAR), a spike in serum cortisol approximately 30 to 45 minutes after awakening. The CAR is important in circadian rhythms and maintaining wakefulness (475). Traditionally, low levels of cortisol are thought to contribute to fatigue (476). Investigation in the context of CFS demonstrates that CFS patients have a decreased CAR and lower levels of serum cortisol compared with non-fatigued individuals (477). However, there are multiple reports of increased levels of cortisol or CRH in people with MS (423-425). The effect of this on wakefulness is not well understood. Smaller studies with a limited number of subjects failed to demonstrate a relationship between cortisol levels and fatigue in MS (450, 478). In contrast, Powell et al. (479) reported an increase in CAR as well as a correlation between fatigue and CAR in patients with RRMS. However, the increased

CAR was not associated with same-day fatigue and cortisol levels were negatively correlated with fatigue in RRMS patients.

While there are several possible explanations for these conflicting results, the most likely explanation is the significant variation between different techniques to measure cortisol. Cortisol has a large circadian variation, with highest levels in the morning and lowest levels around midnight (480). This in itself presents a significant challenge for obtaining accurate measurements. Furthermore, cortisol is transported bound to a specific carrier protein, cortisol binding globulin, as well as albumin. As such, the biologically active fraction of cortisol comprises less than 10% of the total hormone concentration (481). Furthermore, most cortisol is excreted into the urine. Cortisol is usually measured in one of three biological fluids: 1) serum, 2) saliva, and, 3) urine (482). All three methods come with serious limitations and inherent inaccuracies. Serum cortisol can be measured as either total cortisol or free serum. Seriously ill patients often have low plasma protein concentration, making total cortisol analysis difficult (483). Alternatively, assays to measure free cortisol must be very sensitive and there is a lack of standardization between different commercially available kits (484).

There are several methods for measuring salivary cortisol. Salivary measurement has the advantages of not requiring an invasive blood draw, samples can be acquired at a patient's home, and cortisol is stable in saliva at room temperature for at least a week. However, as with serum assays, different salivary cortisol assays each have a different reference range, and there is no certified reference material to compare tests from different laboratories (485). Furthermore, one of the measurements for salivary cortisol must be obtained at midnight, which makes compliance difficult.

Lastly, 24 hour urinary free cortisol has long been used for diagnosing adrenal dysfunction (such as Addison's disease or Cushing syndrome). This technique has the advantage of measuring free cortisol, the active component of the hormone. However, this method is becoming increasingly unpopular due to the methodological concerns of requiring 24 hours of urine collection, as well as suboptimal assay precision (486).

These different methodologies, each with their own strengths and weaknesses, highlight the difficulties in accurately and precisely measuring cortisol. This, along with differences between assay kits and small patient numbers in most MS studies makes formulating conclusions on the role of cortisol in fatigue in MS difficult. A possible solution is the use of hair cortisol. This technique has recently emerged as an accurate, reliable, and noninvasive method of quantifying cortisol (487). Hair cortisol also has the advantage of representing cortisol levels integrated over several months, rather than a single point in time, since cortisol builds up in hair follicles (488). There are currently no studies that assess hair cortisol in MS. This technique may be well suited to monitoring cortisol levels in a chronic disease such as MS, and should be considered in future studies.

2.1.4 Potential causes of hypothalamic dysfunction-induced fatigue

There are many potential mechanisms of hypothalamic dysfunction-induced fatigue. One possible mechanism is leukocyte-mediated damage of neurons within the hypothalamus. Inflammatory lesions detectable by MRI often indicate destruction caused by infiltrating leukocytes. In theory this can lead to altered neuronal signaling, perturbed hormone secretion, or dysfunctional neuropeptide release. This was

highlighted in a recent review by Patejdl et al. (489). In this review the authors hypothesized that the primary cause of fatigue in MS is damage in the brain caused by infiltrating peripheral immune cells. This damage, initiated by leukocyte-derived cytokines, causes neurodegeneration and leads to altered neuronal function and subsequent symptoms. Patejdl et al. also suggested the hypothalamus as a key region where damage can cause fatigue. Specifically, they implicated HPA axis dysfunction, caused by lesions in the hypothalamus, as the driver of fatigue. While literature to support this claim is sparse, there are studies that investigated the correlation between hypothalamic damage and fatigue in MS. For example, Zellini et al. used T1 relaxation time as a measure of pathology in the hypothalamus and found that people with MS had significantly increased T1 relaxation times in the hypothalamus compared with controls, which correlated with overall fatigue score (490). As described above, several case reports describe hypothalamic lesions in MS and symptoms related to neuroendocrine dysfunction, including fatigue. However, there are no publications demonstrating a correlation between the presence of hypothalamic lesions and fatigue.

Another potential cause of fatigue in MS is modulation of HPA axis activity by cytokines derived from leukocytes. The relationship between increased circulating cytokines and fatigue in various diseases is well documented (491-493). Furthermore, individuals with MS are reported to have increased levels of various circulating inflammatory cytokines (494). Heesen et al. (450) investigated the relationship between serum levels of pro-inflammatory cytokines and fatigue in MS and found that levels of TNF-α and IFN-γ were significantly elevated in people with fatigue and correlated with fatigue severity. Furthermore, Malekzadeh et al. (495) reported a positive correlation

between serum IL-6 levels and fatigue, as measured through Checklist Individual Strength, a validated questionnaire to assess chronic fatigue (496). These studies agree with mouse models of inflammatory disease showing that injection of inflammatory cytokines and immune mediators directly into the brain induces HPA axis activation as well as fatigue (124, 457). Additional animal studies are needed to determine if neuroinflammation-induced fatigue depends on HPA axis activation.

In summary, while mechanisms of increased HPA axis activity as well as HPA axis induced fatigue in MS are still not fully understood, it seems that cytokines are key players in this process. Further studies are needed to determine if autoreactive CNS-infiltrating leukocytes are the source of these cytokines, or if HPA axis dysfunction (and subsequent fatigue) is secondary to systemic stress and inflammation (i.e. circulating factors).

3. Weight dysregulation

Another critical function of the hypothalamus is to modulate body composition. Hormones produced in the periphery act on neurons in various nuclei in this region to stimulate or inhibit hunger, as well as control catabolism and anabolism of fat and muscle tissues. Two important weight regulatory hormones produced in the periphery are leptin and ghrelin. Leptin is produced in adipocytes and acts on neurons in the hypothalamus to prevent a starvation response. Ghrelin is produced in the gastrointestinal tract and provides an important stimulatory drive to orexigenic neurons. These hormones act to provide critical inputs to the anorexigenic proopiomelanocortinin neurons and orexigenic neurons co-expressing neuropeptide Y and agouti-related

peptide located in the arcuate nucleus of the hypothalamus. The activity of these neurons is also modulated by cytokines, which can cause disorders of energy balance such as cachexia (267) and obesity (497).

The influence of MS on weight regulation and body composition is not well understood. Khurana et al. reported an increased prevalence of being overweight in VA Veterans with MS (n = 4703) compared to the general average for veterans (42.3% vs. 39.6%, respectively), but a decreased prevalence of being obese (20.1% vs. 33.1%, respectively) (498). A recent study of 130 Israeli MS patients with advanced disease (mean Expanded Disability Status Scale: 5.5) showed that MS patients were less likely to be obese than the general population (499). Other studies investigating the prevalence of obesity in MS also show similar or decreased prevalence in these individuals compared to the general population (500, 501). However, no studies have tracked the BMI of MS patients throughout the disease course and compared that to a similar time period in non-diseased controls. Furthermore, most of these studies used BMI as the only index of body composition. Previous studies show that many people with MS experience muscle mass loss (502-504). This decrease in muscle mass may mask increases in adiposity, making it difficult to assess obesity based on BMI alone. Furthermore, elderly patients and those with chronic disease often suffer from "sarcopenic obesity", where they have decreased muscle mass, yet retain and even increase fat mass (505). Individuals with sarcopenic obesity are at particularly high risk for developing adverse health outcomes (506, 507). While no studies have investigated the prevalence of sarcopenic obesity in MS patients, Pinhas-Hamiel et al. [94] found that while MS patients had lower rates of obesity than the general population, 56% of

individuals with MS had waist circumference consistent with abdominal obesity. This rate is higher than previously reported rates of 21% and 39% in males and females, respectively, in the same age groups in non-diseased individuals. Therefore, it is reasonable to suggest that those with MS are at risk for developing sarcopenic obesity. Future studies should investigate the prevalence and consequences of sarcopenic obesity in people with MS.

Alternatively, another syndrome exacerbated by hypothalamic inflammation is cachexia (121). Cachexia is a metabolic syndrome with cardinal features of anorexia, weight loss (with disproportional loss of lean mass), and fatigue (30). It occurs in numerous chronic diseases, such as cancer (508), HIV (509), cirrhosis (510), and Alzheimer's disease (511). Systemic inflammation is a key component of cachexia, and is associated with elevated serum levels of inflammatory cytokines such as TNF-α, IL-6, and IL-1β, similar to MS. Furthermore, cachexia is prevalent in autoimmune diseases, especially rheumatoid arthritis (512). Therefore, it is reasonable to suggest that people with MS are vulnerable to cachexia. However, cachexia in MS is not well studied. A recent case report described an MS patient with lateral hypothalamic lesions and cachexia (513). Furthermore, cachexia is noted as a common cause of death in patients with advanced MS (514) and thus may be a concern in advanced disease. Unfortunately, no studies investigated the prevalence of cachexia at any point in the disease. Further studies are needed to determine the presence and role of this syndrome in the clinical course and pathophysiology of MS.

5. Treatments

Treatments for hypothalamic dysfunction in MS are not well defined. There are currently no FDA-approved treatments specifically for fatigue or weight dysfunction in MS. However, there are a few pharmacologic agents that are used for fatigue in MS that in small-scale studies have demonstrated moderate efficacy. The two most frequently used are Amantadine and Modafinil. Amantadine was originally used for influenza but now is most commonly used in Parkinson's disease. Its mechanism of action is not well understood. Although it is the most frequently studied medication for fatigue in MS, few placebo-controlled double-blinded studies are published, and all contain fewer than 125 subjects (515-517). Nevertheless, these studies show a small but statistically significant benefit compared to placebo. However, a Cochrane Review determined that these studies were of poor quality and vulnerable to bias (518). Additional clinical and mechanistic studies are needed to fully understand the efficacy of Amantadine.

Modafinil is a commonly used therapy for promoting wakefulness in various states of fatigue and sleep dysfunction (519). While its mechanism is also not fully understood, it is thought to prevent reuptake of monoamines, including dopamine, norepinephrine, and serotonin (520). As with Amantadine, studies investigating the effects of Modafinil on fatigue in MS are weak and underpowered (521), but suggest that Modafinil treatment modestly improves fatigue (522, 523). However, a recent meta-analysis showed that exercise and education are as effective as Amantadine or Modafinil (524).

Stimulants such as Methylphenidate have been proposed as third-line treatment for fatigue in MS (525) , but there a currently no published clinical trials to support their use. Alternative treatments proposed for fatigue in MS include high dose aspirin, ginkgo

biloba, and amino acid supplements (526), but these treatments are seldom used and evidence supporting their efficacy is weak to non-existent.

There are currently no approved therapies for weight dysregulation in MS. Since this issue is still poorly understood, few therapies have been studied or proposed. The only studies published focus on therapeutic use of cannabis. These studies are few, underpowered, and lack control for confounders (527, 528). Zajicek et al. (528) reported that perceived increased appetite (but not actual food intake) was reported at low levels in the cannabis group. Additional research on the prevalence and mechanisms of weight and appetite dysregulation in MS are needed prior to treatment development.

None of the current therapies for fatigue target hypothalamus-based or other established or hypothesized mechanisms of fatigue in MS. Future clinical studies to develop therapies for fatigue in MS should focus on established or proposed mechanisms in the CNS. Animal models can provide a foundation for these studies. As described in Section 2.1.2, the orexin system can be targeted pharmacologically. Due to the diurnal nature of orexin secretion, agonist use during the day and/or antagonist use at night should be considered. For example, administration of orexin to mice with EAE greatly attenuated clinical symptoms (529). However, fatigue was not directly measured in this study. Alternatively, orexin antagonists were proposed as treatment for insomnia (530, 531). Currently, Suvorexant, the dual orexin receptor antagonist (DORA), is the only FDA-approved orexin antagonist therapy available. In a phase III trial of 1,260 patients with insomnia, individuals in the treatment group (n = 493) experienced prolonged sleep maintenance and decreased sleep onset compared to placebo controls

(n = 767) (532). The relationship between insomnia and fatigue in MS must be better understood in order to justify use of DORAs.

6. Future Directions

Future research investigating hypothalamic dysfunction in MS should focus on accurately and reproducibly measuring hypothalamic hormones and neuropeptides, HPA axis activity, and structural integrity of the hypothalamus in MS patients. Large multi-centered trials should be conducted in order to obtain a sufficient number of patients. Subjects should be selected based on disease stage and groups should be as clinically homogenous as possible. Furthermore, since there are considerable differences in hypothalamic function between men and women (533), studies should include both genders and consider sex as a key variable. These studies will help lay a stronger foundation to determine the prevalence and typical severity of hypothalamic involvement in MS.

Additional imaging studies focused on hypothalamic lesions should be conducted to accurately determine the prevalence of lesions in this brain region in MS. Furthermore, correlations between hypothalamic lesions, perturbed hormone and neuropeptide secretion, and signs and symptoms (fatigue, sleep disturbances, etc.) are needed to determine whether hypothalamic dysfunction in MS is due to structural damage in this region or other causes.

Lastly, very few studies have investigated the effect of MS on weight homeostasis or body composition. Future studies should investigate both obesity and cachexia. MS shares many characteristics of other diseases that have a high

prevalence of cachexia (e.g. high levels of circulating inflammatory cytokines, inflammation in the CNS, HPA axis hyperactivity, etc.), yet no studies investigated the prevalence of cachexia in MS. Alternatively, more detailed measurements of body composition are needed to determine if individuals with MS have increased adiposity and/or an increased prevalence of sarcopenic obesity.

7. Conclusion

The hypothalamus is the central regulator of numerous homeostatic processes. In MS, many of these processes, especially wakefulness and activity level, are disrupted. This can lead to debilitating symptoms such as fatigue. However, the hypothalamus and its functions are often overlooked in MS. The few studies investigating hypothalamic dysfunction in MS show dysfunctional secretion of hypothalamic peptides and hormones in MS patients, including cortisol and orexin. Results are conflicting and studies are underpowered. In addition, MS is similar to other diseases that have a high prevalence of cachexia, a syndrome caused by hypothalamic dysfunction. However, since obesity is certainly more common than cachexia, understanding the link between MS and obesity should be considered a priority. Further studies consisting of larger, more homogenous patient samples are needed to fully understand the degree and role of hypothalamic dysfunction in MS. These studies can provide a basis for additional treatments, as hypothalamic hormones and neuropeptides present substrates that can be targeted pharmacologically.

Chapter 3: TRIF is a Key Inflammatory Mediator of the Acute Sickness Response and Cancer Cachexia.

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Title: "TRIF is a key inflammatory mediator of acute sickness behavior and cancer cachexia"

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Highlights

- TRIFKO mice have attenuated sickness behavior after LPS exposure.

- TRIF is important for neutrophil recruitment and microglia activation after central LPS exposure.

- TRIFKO mice have attenuated anorexia, muscle catabolism, and fatigue during cancer.

Abstract

Hypothalamic inflammation is a key component of acute sickness behavior and cachexia, yet mechanisms of inflammatory signaling in the central nervous system remain unclear. Previous work from our lab and others showed that while MyD88 is an important inflammatory signaling pathway for sickness behavior, MyD88 knockout (MyD88KO) mice still experience sickness behavior after inflammatory stimuli challenge. We found that after systemic lipopolysaccharide (LPS) challenge, MyD88KO mice showed elevated expression of several cytokine and chemokine genes in the hypothalamus. We therefore assessed the role of an additional inflammatory signaling pathway, TRIF, in acute inflammation (LPS challenge) and in a chronic inflammatory state (cancer cachexia). TRIFKO mice resisted anorexia and weight loss after peripheral (intraperitoneal, IP) or central (intracerebroventricular, ICV) LPS challenge and in a model of pancreatic cancer cachexia. Compared to WT mice, TRIFKO mice showed attenuated upregulation of *Il6*, *Ccl2*, *Ccl5*, *Cxcl1*, *Cxcl2*, and *Cxcl10* in the hypothalamus after IP LPS treatment, as well as attenuated microglial activation and neutrophil infiltration into the brain after ICV LPS treatment. Lastly, we found that TRIF was required for *Ccl2* upregulation in the hypothalamus and induction of the catabolic genes, *Mafbx*, *Murf1*, and *Foxo1* in gastrocnemius during pancreatic cancer. In summary, our results show that TRIF is an important inflammatory signaling mediator of sickness behavior and cachexia and presents a novel therapeutic target for these conditions.

Keywords: Cachexia, neuroinflammation, hypothalamus, sickness behavior, neuroimmunology, microglia

1. Introduction

Innate immune activation in response to various pathogens leads to systemic and central nervous system (CNS) inflammation, inducing a distinct metabolic and behavioral paradigm that includes fever, weight loss, anorexia, and fatigue. This constellation of signs and symptoms, referred to as "sickness behavior" (1), is critical for combating infection and allows resources to be diverted to the immune system to fight pathogens. However, if sickness behavior is maintained in conditions of chronic inflammation, it can become maladaptive and manifest as cachexia. Cachexia is a devastating syndrome characterized by anorexia, increased catabolism of lean body mass, and lethargy (30, 534, 535). It is prevalent in numerous chronic diseases, including cancer (508), chronic renal failure (536), congestive heart failure (537), and untreated HIV (538). Furthermore, cachexia is associated with increased mortality of the underlying disease and decreased quality of life (539-541). Despite this serious clinical concern, there are currently no effective treatments and mechanisms remain controversial.

Our lab, along with others, described a CNS-based mechanism of cachexia in which cytokines generated in the periphery are amplified and modified within the hypothalamus, leading to aberrant activity of weight- and activity-modulating neurons (124, 542, 543). Specifically, intracerebroventricular (ICV) injection of inflammatory cytokines (252, 255) or pathogen associated molecular patterns such as lipopolysaccharide (LPS) (270) potently reduces food intake and activity. Furthermore, peripheral or central cytokine injection or immune challenge leads to rapid activation of neurons in areas that are critical for food intake and energy metabolism, such as the

nuclei of the mediobasal hypothalamus (MBH) (300, 544-546). However, the cellular and molecular pathways whereby peripheral inflammation is translated in the brain into behavioral or metabolic responses are still not well understood.

Toll-like receptors (TLRs) are key components of the innate immune system, recognizing a variety of pathogens and inflammatory signals. TLR function is important for mounting an appropriate inflammatory response, and metabolic signaling in the CNS is closely tied to TLR signaling (162). Pro-inflammatory signaling via the Myeloid Differentiation Primary Response Gene 88 (MyD88) pathway was initially thought to be the dominant mechanism whereby the binding of pathogenic signaling molecules to receptors is linked to the synthesis and release of inflammatory cytokines and chemokines (547). However, recent data suggest that MyD88-independent pathways linking TLRs to cellular activation are present within the brain (548, 549). In our previous work, we found that while sickness behavior was severely attenuated in MyD88KO mice, a slight anorexia response was present and muscle catabolism occurred (550). The adaptor protein TIR-domain-containing adaptor inducing interferon-β (TRIF) is an important inflammatory signaling mediator, yet has received little attention in the context of CNS-mediated alterations in behavior and metabolism during illness. TRIF is the dominant adapter for TLR3 signaling, and plays an essential role in TLR4 responses to LPS as well (551). Furthermore, TRIF knockout (TRIFKO) mice are nearly as resistant to endotoxin-induced mortality as are MyD88KO mice (552).

The role of TRIF signaling in the CNS during acute sickness behavior and cachexia is unknown. We found that TRIF signaling is important for neuroinflammation and resulting acute sickness behavior after systemic or central exposure to LPS. We also

found that mice lacking TRIF have attenuated cancer cachexia. These results implicate TRIF as a key signaling mediator in inflammation-driven behavioral and metabolic changes during illness, and a potential therapeutic target for cachexia.

2. Materials and Methods

2.1 Animals

Male and female 20–25-g WT C57BL/6J (stock no. 000664), MyD88KO (stock no. 009088), TRIFKO (Trif^{Lps2}, stock no. 005037) mice were obtained from The Jackson Laboratory. No immune, developmental, or behavioral abnormalities were observed in TRIFKO mice by our group, consistent with the existing literature (133, 551). Furthermore, they consume the same amount of food as WT animals in the absence of inflammation (see Figs 2 and 3), and their spleens are the same size (data not shown). Mice were between 7 and 12 weeks of age at time of experiment. All animals were maintained at 27°C on a normal 12:12 hr light/dark cycle and provided *ad libitum* access to water and food. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Oregon Health and Science University.

2.2 Intracerebroventricular Cannulation and Injections

Mice were anesthetized under isoflurane and placed on a stereotactic alignment instrument (Kopf Instruments, CA). 26-gauge lateral ventricle cannulas were placed at −1.0 mm X, −0.5 mm Y, and −2.25 mm Z relative to bregma. Injections were given in 2 µl total volume. LPS (from *Escherichia coli*, O555:B5, Sigma Aldrich, St. Louis, MO) was dissolved in normal saline with 0.5% bovine serum albumin.

2.3 Nocturnal Feeding Studies

Animals were transferred to clean cages and injected with ICV (50 ng) or IP (250 µg) LPS 1 h prior to lights off. At 2, 6, 12, 24, 36 and 48 hrs after the onset of the dark cycle, food was weighed and returned to the cage. Body weight was recorded at 12, 24, 36, and 48 hrs.

2.4 Plasma Corticosterone Measurement

Plasma corticosterone levels were measured by RIA (MP Biomedicals, Valiant, Yantai, China) according to the manufacturer's instructions. Animals were anesthetized with a lethal dose of a ketamine/xylazine/acetapromide 4 hrs after IP LPS administration. Blood was obtained by cardiac puncture, anticoagulated with EDTA and separated by centrifugation. Plasma was stored at −80°C until analysis.

2.5 Quantitative Real-Time PCR

Prior to tissue extraction, mice were euthanized with a lethal dose of a ketamine/xylazine/acetapromide and sacrificed. Hypothalamic blocks were dissected, snap frozen, and stored in −80 °C until analysis. Hypothalamic RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was transcribed using TaqMan reverse transcription reagents and random hexamers according to the manufacturer's instructions. PCR reactions were run on an ABI 7300 (Applied Biosystems, Foster City, CA), using TaqMan universal PCR master mix with the following TaqMan mouse gene expression assays, selected based on previous studies from our lab and others which showed their importance in sickness behaviors and cachexia (124, 133, 553): *18s* (Mm04277571_s1), *Il1b*

(Mm00434228_m1), *Tnf* (Mm00443258_m1), *Il6* (Mm01210732_g1), *Cd80* (Mm00711660_m1), *Myd88* (Mm00440338_m1), *Ifnβ (*Mm00439552_s1), *Ccl2* (Mm99999056_m1), *Ccl5* (Mm01302427_m1), *Cxcl1* (Mm04207460_m1), *Cxcl2* (Mm00436450_m1), *Cxcl10* (Mm00445235_m1), *Gapdh* (Mm99999915_g1), *Mafbx* (Mm00499518_m1), *Murf1* (Mm01185221_m1), and *Foxo1* (Mm00490672_m1). It is important to note that *Ifnβ* is a single exon gene and therefore results may be confounded by genomic DNA. However, we used DNAse to eliminate genomic DNA and also ran a no-primer control, which showed no amplification (not shown).

Relative expression was calculated using the ΔΔCt method and normalized to WT vehicle treated or sham control. Statistical analysis was performed on the normally distributed ΔCt values.

2.6 Immunohistochemistry

Mice were anesthetized using a ketamine/xylazine/acetapromide cocktail and sacrificed by transcardial perfusion fixation with 15 mL ice cold 0.01 M PBS followed by 25 mL 4% paraformaldehyde (PFA) in 0.01 M PBS. Brains were post-fixed in 4% PFA overnight at 4°C and cryoprotected in 20% sucrose for 24 hrs at 4°C before being stored at −80°C until used for immunohistochemistry. Immunofluorescence histochemistry was performed as described below. Free-floating sections were cut at 30 μm from perfused brains using a sliding microtome (Leica SM2000R, Leica Microsystems, Wetzlar, Germany). Hypothalamic sections were collected from the division of the optic chiasm (bregma −1.0 mm) caudally through the mammillary bodies (bregma −3.0 mm). Sections were incubated for 30 min at room temperature in blocking

reagent (5% normal donkey serum in 0.01 M PBS and 0.3% Triton X-100). After the initial blocking step, sections were incubated in rabbit anti-mouse Iba-1 (1:500, DAKO) in blocking reagent for 24 hrs at 4°C, followed by incubation in donkey anti-rabbit Alexa 555 (1:1000) for 2 hrs at room temperature. Between each stage, sections were washed thoroughly with 0.01 M PBS. Sections were mounted onto gelatin-coated slides and coverslipped using Prolong Gold Antifade media with DAPI (Thermofisher, Waltham, MA).

2.7 Microglia Activation Quantification

Microglia activation in the MBH was quantified using Fiji (ImageJ, NIH, Bethesda, MD). The MBH was defined as the region surrounding the third ventricle at the base of the brain, starting rostrally at the end of the optic chiasm when the arcuate nucleus appears (-1.22 mm from bregma) and ending caudally at the mammillary body (-2.70 mm from bregma). Images were acquired using the 20X objective (na=0.8, step size=1 μm) with a 10X ocular zoom, resulting in 200X magnification. The base of the MBH was positioned at the very bottom of the field of view (FOV) and the third ventricle at the center of the FOV. Care was taken to exclude the meninges so as to avoid analysis of meningeal macrophages. Images were 2048 x 2048 pixels, with a pixel size of 0.315 μm. Images were acquired as 8-bit RGB TIFF images. 3-10 MBH images per animal were acquired and analyzed by a researcher blinded to genotype and treatment group (KGB).

After image acquisition, TIFF images were uploaded to Fiji and converted to 8-bit greyscale images. After thresholding, microglia were identified using the Analyze Particle function, which measured mean Iba-1 fluorescent intensity per cell and cell area.

Iba-1 fluorescent intensity and cell size was measured for each microglia in the arcuate

nucleus. Due to the density of microglia in the median eminence (ME), the software was

unable to differentiate individual cells. As such, overall Iba-1 fluorescent intensity was

measured to quantify microglia activation in the ME.

2.8 Flow Cytometry

12 hrs after 500 ng ICV LPS administration, mice were anesthetized using a

ketamine/xylazine/acetapromide cocktail and perfused with 15 mL ice cold 0.01 M PBS

to remove circulating leukocytes. After perfusion, brains were extracted and minced in a

digestion solution containing 1 mg/mL type II collagenase (Sigma) and 1% DNAse

(Sigma) in RPMI, then placed in a 37°C incubator for 1 hr. After digestion, myelin was

removed via using 30% percoll in RPMI. Isolated cells were washed with RPMI,

incubated in Fc block for 5 min, then stained with the following antibodies (all rat anti-

mouse from BioLegend, except for Live/Dead) (BioLegend, San Diego, CA): anti-CD45

PerCP/Cy5.5 (1:400), anti-CD11b APC (1:800), anti-Ly6C PerCP (1:100), anti-Ly6G

PE/Cy7 (1:800), anti-CD3 PE (1:100), and Live/Dead fixable aqua (1:200, Thermofisher).

Flow cytometry was conducted using a Fortessa analytic flow cytometer (BD

Biosciences, NJ), and analysis was performed on FlowJo V10 software (FlowJo,

Ashland, OR). Cells were gated on LD, SSC singlet, and FSC singlet (Fig. 3 – figure

supplement 1). Leukocytes were then defined as CD45+ cells and identified as either

peripheral myeloid cells (CD45^{high}CD11b+) or lymphocytes (CD45^{high}CD11b-). From

peripheral myeloid cells Ly6C^{low} monocytes (Ly6C^{low}Ly6G-), Ly6C^{high} monocytes

(Ly6C^{high}Ly6G-), and neutrophils (Ly6C^{mid}Ly6G+) were identified. From lymphocytes, CD3+ cells were identified as T-cells.

2.9 KPC Cancer Cachexia Model

Our lab generated a mouse model of pancreatic ductal adenocarcinoma (PDAC) – associated cachexia by injection of murine-derived KPC PDAC cells (originally provided by Dr. Elizabeth Jaffee from Johns Hopkins) (122). These cells are derived from tumors in mice with KRAS^{G12D} and TP53^{R172H} deleted via the PDX-1-Cre driver (554). Cells were maintained in RPMI supplemented with 10% heat-inactivated FBS, and 50 U/mL penicillin/streptomycin (Gibco, Thermofisher, Waltham, MA), in incubators maintained at 37°C and 5% CO$_2$. In the week prior to tumor implantation, animals were transitioned to individual housing to acclimate to experimental conditions. Animal food intake and body weight were measured once daily. Mice were inoculated orthotopically with 3 million KPC tumor cells in 40 μL PBS into the tail of the pancreas (555). Sham-operated animals received heat-killed cells in the same volume. Nuclear Magnetic Resonance (NMR) measurements were taken at the beginning of the study for covariate adaptive randomization of tumor and sham groups to ensure equally distributed weight and body composition. Voluntary home cage locomotor activity was measured via MiniMitter tracking devices (Starr Life Sciences, Oakmont, PA). We only analyzed dark phase activity, based on the nocturnal nature of mice (556) and our previous studies that revealed little activity during the light phase (122), most of which was likely caused by humans entering the room. Mice were implanted 7 days prior to tumor implantation with MiniMitter transponders in the intrascapular subcutaneous space. Using these devices,

movement counts in *x*- axis, *y*- axis, and *z*- axis were recorded in 5 min intervals. Bedding was sifted daily for any chow that may have fell to the cage floor. At 10 days post inoculation, a point when animals consistently develop cachexia (122), animals were euthanized, and tissues were extracted for analysis.

2.10 Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed with Prism 7.0 software (Graphpad Software Corp, La Jolla, CA). All data were analyzed with Two-way ANOVA analysis. For single time point experiments, the two factors in ANOVA analysis were genotype (WT vs TRIFKO in all experiments) or treatment (Saline vs LPS in Figs 1-4 or Sham vs Tumor in Fig 5). In repeated measures experiments (time course experiments – Figs 2A and B, 3A and B, 5A and C), the two factors were group and time. Main effects of genotype, treatment, group, time, and/or interaction were first analyzed, and if one effect was significant, *Bonferroni* post hoc analysis was then performed. For all analyses, significance was assigned at the level of $p < 0.05$.

3. Results

3.1 MyD88KO mice experience hypothalamic inflammation after systemic LPS challenge.

Based on our previous work showing that MyD88KO mice still show subtle signs of sickness behavior after systemic LPS challenge (slight anorexia, muscle catabolism, and induction of catabolic genes) (550), we hypothesized that hypothalamic inflammation also occurred in these mice. We found that 6 hrs after 250 μg/kg IP LPS, MyD88KO mice showed increased expression of several cytokine and chemokine genes in the hypothalamus, including *Il1β*, *Tnf*, *Il6*, *Ifnβ*, *Ccl2*, *Ccl5*, *Cxcl1*, *Cxcl2*, and *Cxcl10* (Fig. 1) (Two way ANOVA treatment effect all at least p<0.001, post hoc analysis of MyD88KO saline vs. MyD88KO LPS all at least p<0.01). While upregulated in LPS-treated WT mice, *Cd80* was not significantly upregulated in LPS-treated MyD88KO mice (treatment $F_{1,12}$ = 23.28, p<0.001; genotype $F_{1,12}$ = 15.14, p=0.002; interaction $F_{1,12}$ = 19.16, p<0.001. WT saline vs. WT LPS p<0.001, MyD88KO saline vs. MyD88KO LPS p>0.99 in post hoc analysis). It is important to note that the vast majority of LPS-induced cytokine and chemokine gene upregulation in the hypothalamus was attenuated in MyD88KO mice. Interestingly, *Ifnβ* only showed a slight non-significant increase in LPS-treated WT mice compared to saline-treated WT mice, yet showed a 3.9-fold upregulation in LPS-treated MyD88KO mice compared to saline-treated MyD88KO mice (treatment effect $F_{1,12}$=8.83, p=0.01, WT saline vs. WT LPS p=0.75. MyD88KO saline vs. MyD88KO LPS p=0.01 in post hoc analysis). Basal expression of all cytokines/chemokines was detectable in hypothalami of saline-treated animals.
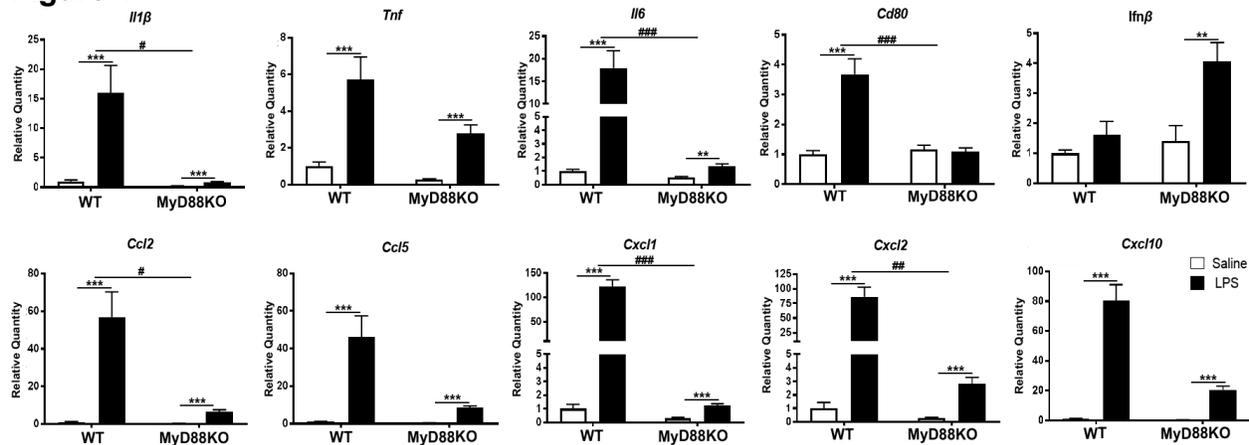
Figure 1

Figure 1: Mice lacking MyD88 experience hypothalamic inflammation after systemic LPS challenge. Expression of inflammatory cytokine genes in the hypothalamus 6 hrs after 250 µg/kg IP LPS treatment. ** = $p < 0.01$, *** = $p < 0.001$ for Two-way ANOVA Bonferroni post hoc comparisons comparing saline-treated animals to LPS-treated animals within the same genotype. # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$ for interaction effect in Two-way ANOVA. N = 3-5/group.

3.2 Mice lacking TRIF show attenuated acute illness response after systemic LPS challenge

Since MyD88KO mice still show sickness behavior and hypothalamic inflammation after systemic LPS challenge, we hypothesized that other inflammatory signaling pathways are important for TLR4-driven sickness behavior. TRIF is an important adaptor protein for innate immune activation (551), yet its role in sickness behavior after LPS challenge is unknown. After systemic LPS challenge (250 µg/kg, IP), TRIFKO mice showed attenuated anorexia (group effect $F_{3,140} = 312.9$, $p < 0.001$, post hoc analysis of WT LPS vs. TRIFKO LPS at least $p < 0.001$ every time point after LPS injection) and weight loss (group effect $F_{3,100} = 431.7$, $p < 0.001$, post hoc analysis of WT LPS vs. TRIFKO

LPS at least p<0.01 starting 6 hrs after LPS injection) compared to WT mice (Fig. 2A and B). Next, in order to determine the degree of hypothalamic activation and quantify acute stress response, we measured plasma corticosterone (557). While WT mice showed a large increase in plasma corticosterone 4 hrs after IP LPS administration (p<0.001), LPS-treated TRIFKO mice did not show a significant increase (interaction effect F1,18 = 19.79, p <0.001, WT saline vs. WT LPS p<0.01, TRIFKO saline vs. TRIFKO LPS p = 0.06 in post hoc analysis)(Fig. 2C).

CNS inflammation is a hallmark of acute illness responses and cachexia. Therefore, we measured expression of inflammatory cytokine and chemokine genes in the hypothalamus after systemic LPS challenge using qRT-PCR. We found that 6 hrs after 250 μg/kg IP LPS, TRIFKO animals showed attenuated up-regulation of several cytokines and chemokines in the hypothalamus, including *Il6*, *Ccl2*, *Ccl5*, *Cxcl1*, *Cxcl2*, and *Cxcl10* (interaction effect at least p<0.05) (Fig. 2D). Alternatively, *Il1β*, *Tnf* were not differentially upregulated in LPS-treated TRIFKO mice compared to WT LPS-treated mice (treatment effect p<0.05, and post hoc analysis comparing saline to LPS-treatment within the same genotype p<0.05, but interaction effect p>0.05 for both). In contrast to Figure 1, we did not observe a treatment effect of LPS on WT mice for *Cd80* (treatment effect F1,12 = 0.68, p=0.43), yet did see an interaction effect (F1,12 = 6.57, p=0.02). Lastly, we did not observe any treatment (F1,12 = 0.726, p=0.411), genotype (F1,12 = 0.346, p=0.567), or interaction (F1,12<0.001, p=0.995) effect in our analysis of *Ifnβ* expression in the hypothalamus. It is important to note that basal expression of all cytokines/chemokines was detectable in hypothalami of saline-treated animals.

In order to rule out altered MyD88 signaling as a result of TRIF deletion, we challenged TRIFKO mice with 10 ng ICV IL-1β. MyD88 is essential for IL-1R signaling, but TRIF is not involved (558). We found that WT and TRIFKO mice had similar anorexia response to ICV IL-1β (Fig. S1A). While WT IL-1β-treated mice lost more weight than WT saline-treated mice, it was not significantly more than TRIFKO IL-1β-treated mice. Lastly, *Myd88* was equally expressed in WT and TRIFKO mice at baseline, and similarly upregulated after IP LPS exposure (Fig. S1C).

Figure 2

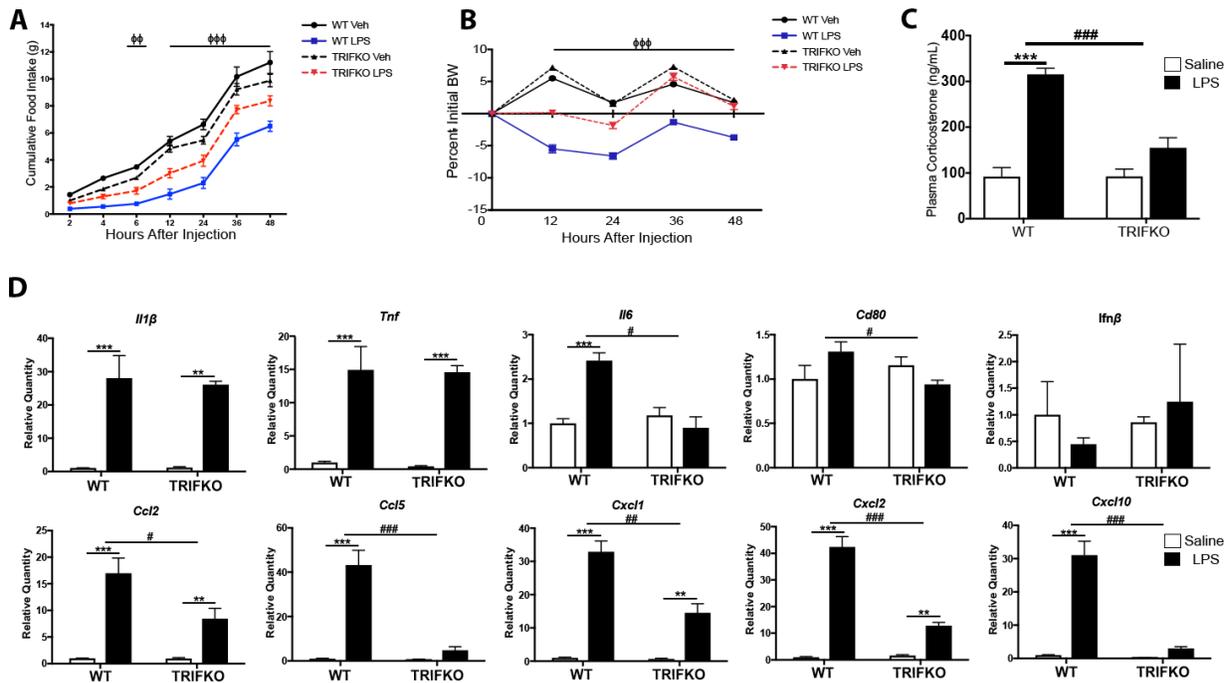Figure 2: **TRIFKO mice have attenuated acute sickness behavior in response to systemic LPS exposure.** A) Cumulative food intake after 250 µg/kg IP LPS treatment. Veh = vehicle treatment (BSA/saline). ΦΦ = p<0.01 for WT LPS vs. TRIFKO LPS in Bonferroni post hoc comparisons at 6 hrs after injection, ΦΦΦ = p<0.001 for WT LPS vs. TRIFKO LPS in Bonferroni post hoc comparisons at 12, 24, 36, and 48 hrs after

injection. B) Body weight change after 250 µg/kg IP LPS treatment. BW = body weight. ΦΦΦ = p<0.001 for WT LPS vs. TRIFKO LPS in Bonferroni post hoc comparisons at 12, 24, 36, and 48 hrs after injection. N = 5-7/group. C) Plasma corticosterone measurement 4 hrs after 250 µg/kg IP LPS treatment. N = 5/group. *** = p<0.001 for Bonferroni post hoc comparisons comparing saline-treated animals to LPS-treated animals within the same genotype, ### = p<0.001 for interaction effect in Two-way ANOVA. D) Expression of inflammatory cytokine genes in the hypothalamus 6 hrs after 250 µg/kg IP LPS treatment. All data are analyzed from ΔCt values and normalized to WT saline group. ** = p<0.01, *** = p<0.001 for Bonferroni post hoc comparisons comparing saline-treated animals to LPS-treated animals within the same genotype. # = p<0.05, ## = p<0.01, ### = p<0.001 for interaction effect in Two-way ANOVA. N = 3-4/group. One outlier was removed in the TRIFKO LPS groups due to complete lack of behavioral response to LPS.

3.3 TRIF is important in acute illness response after ICV LPS challenge

To determine the role of TRIF signaling in the CNS after TLR4 activation, we injected LPS directly into the brain lateral ventricles of WT and TRIFKO mice at a dose that has no behavioral effects when injected peripherally (50 ng). Two-way ANOVA analysis revealed a significant group effect after ICV injection of 50 ng LPS ($F_{3,126}$ = 313.4, p<0.001). Subsequent post hoc *analysis* showed that while LPS caused a significant decrease in cumulative food intake in both WT and TRIFKO animals, starting 36 hrs after treatment TRIFKO animals consumed more than WT animals (p at least <0.01) (Fig. 3A). We found similar results when comparing body weight after ICV LPS

treatment. Two-way ANOVA analysis revealed a significant group effect after ICV injection of 50 ng LPS ($F_{3,90}$ = 103.2, p<0.001), and subsequent post hoc analysis showed that TRIFKO mice treated with ICV LPS experienced significantly attenuated weight loss compared to WT mice treated with ICV LPS at 24 and 36 hrs after injection (p at least <0.01)(Fig. 3B).

3.4 TRIF is required for microglial activation after ICV LPS treatment

TRIF is important for microglia function during states of disease (559). However, no studies have investigated the role of TRIF in microglial activation after TLR4 stimulation. Therefore, we quantified microglial activation in the MBH 12 hrs after ICV LPS administration (50 ng) by measuring Iba-1 intensity per cell and cell area. While arcuate nucleus microglia in LPS-treated WT mice showed a significant increase in size compared to saline-treated WT, arcuate nucleus microglia in LPS-treated TRIFKO mice did not increase in size compared to saline-treated TRIFKO mice (treatment effect $F_{1,12}$ = 16.73, p = 0.0015, WT saline vs. WT LPS p<0.01, TRIFKO saline vs. TRIFKO LPS p = 0.32 in post hoc analysis) (Fig. 3E). In the arcuate nucleus, Iba-1 intensity per microglia did not increase in the LPS-treated group for either genotype (Fig. 3F). However, overall Iba-1 intensity increased in the median eminence in the WT LPS-treated group compared to the WT saline-treated group, but not in the TRIFKO LPS-treated group compared to the TRIFKO saline-treated group (treatment effect $F_{1,12}$ = 8.82, p = 0.02, WT saline vs. WT LPS p<0.05, TRIFKO saline vs. TRIFKO LPS p>0.99 in post hoc analysis) (Fig. 3G).

Figure 3
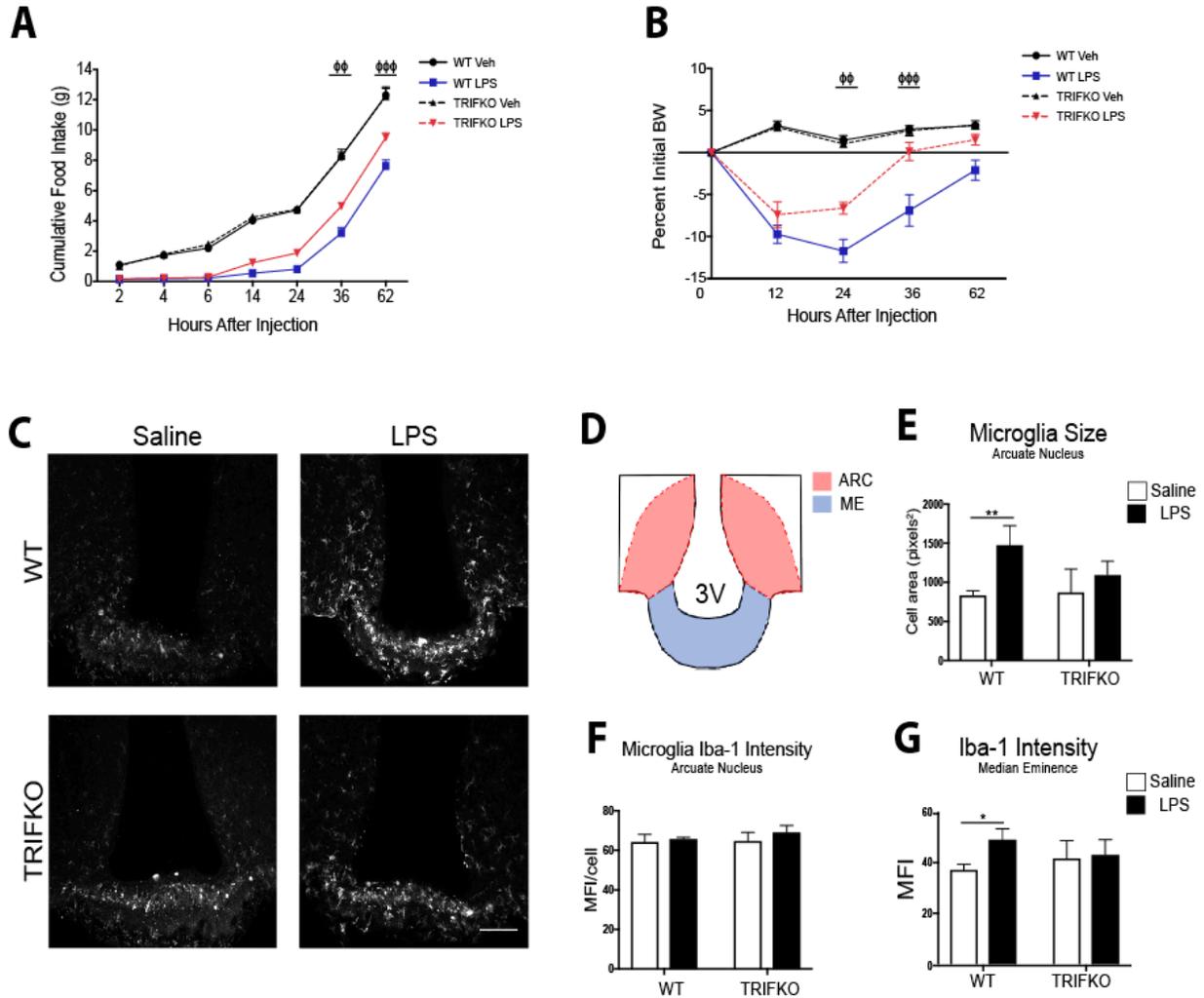
Figure 3: TRIFKO mice have attenuated acute sickness behavior in response to central nervous system LPS exposure. A) Cumulative food intake after 50 ng ICV LPS treatment. Veh = vehicle treatment. B) Body weight change after 50 ng ICV LPS treatment. BW = body weight. For A and B ΦΦ = p<0.05, ΦΦΦ = p<0.001 for WT LPS vs. TRIFKO LPS in Two-Way ANOVA Bonferroni post hoc comparisons. N = 5-6/group. C) Representative images of Iba-1 immunoreactivity in 200X magnification images of the MBH in WT and TRIFKO mice after either 50 ng ICV LPS or saline. Scale bar = 100 μm. D) Cartoon of MBH depicting regions of interest for quantification. ARC = arcuate

nucleus, ME = median eminence. E) Quantification of arcuate nucleus microglia size (area) in pixels^2 after either ICV LPS or saline. F) Quantification of Iba-1 intensity per microglia in the arcuate nucleus in WT and TRIFKO mice after either ICV LPS or LPS saline. G) Quantification of Iba-1 intensity in the median eminence in WT and TRIFKO mice after either ICV LPS or LPS saline. For E-G * = p<0.05, ** = p<0.01 for Bonferroni post hoc comparisons comparing saline-treated animals to LPS-treated animals within the same genotype. n = 4/group.

3.5 TRIF is required for neutrophil recruitment to the brain

Since chemokines comprised the majority of inflammatory transcripts that were less upregulated in TRIFKO mice after LPS exposure, we hypothesized that TRIF is important in immune cell recruitment to the brain. We performed flow cytometry on the brains of WT and TRIFKO mice 12 hrs after 500 ng ICV LPS exposure. We focused our analysis on neutrophils based on previous literature showing they are the predominant cell type in the brain after LPS exposure (232). We found that, compared to saline-treated WT mice, LPS-treated WT mice had a significantly higher percentage of CD45+ cells in the brain that were neutrophils (treatment effect F1,12 = 18.69, p = 0.001, WT saline vs. WT LPS p = 0.0028 in post hoc analysis) (Fig. 4C). Alternatively, compared to saline-treated TRIFKO mice, LPS-treated TRIFKO mice did not have an increased percentage of CD45+ cells in the brain that were neutrophils (TRIFKO saline vs. TRIFKO LPS p = 0.14) (Fig. 4C). In WT animals there was a decrease in percentage of CD45+ cells that were T-cells (treatment effect F1,12 = 8.15, p=0.0145, WT saline vs WT LPS p = 0.019 in post hoc analysis). There was no change in Ly6C^{low}, or Ly6C^{high}

monocytes after LPS exposure in either genotype (treatment, genotype, and interaction all p>0.05 in Two-way ANOVA) (Fig. 4D).

Figure 4

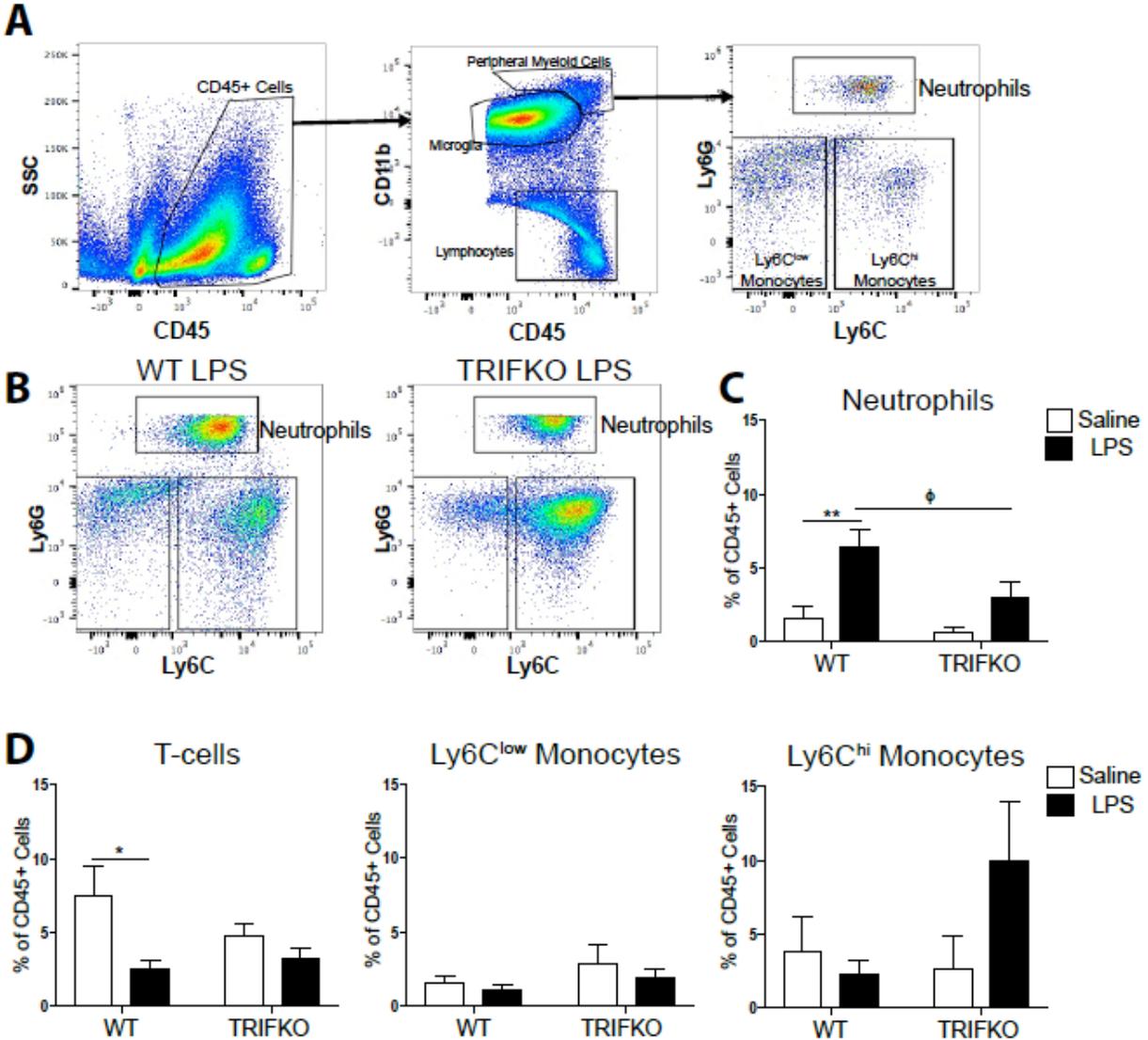**Figure 4: TRIF is required for neutrophil recruitment to the brain after ICV LPS.** A) Flow cytometry gating strategy for various immune cell types in the brain from representative WT brain treated with ICV saline. B) Representative flow cytometry plots from WT and TRIFKO brains treated with 500 ng ICV LPS, gated for CD45^{high}CD11b+ myeloid cells. Note that the population defined as neutrophils (Ly6C^{mid}Ly6G^{high}) may

appear compressed in the TRIFKO LPS plot. This is due to the wide range of Ly6G expression on neutrophils, and a nonsignificant increase in Ly6G expression in brain-infiltrating neutrophils in LPS-treated TRIFKO mice (Figure S3). As a result, some neutrophils reached the pre-defined "maximum" fluorescent intensity ($10^{5.5}$). Any cells with a Ly6G fluorescent intensity greater than this were assigned a fluorescent intensity of $10^{5.5}$. As shown in Figure S3, all such events were still counted as "neutrophils" in quantitative analysis. C) Quantification of neutrophils in the brain as percentage of total CD45+ in WT and TRIFKO brains treated with either ICV LPS or saline. ** = $p<0.01$ for comparing saline-treated to LPS-treated animals within the same genotype, Φ = $p <0.05$ for WT LPS vs. TRIFKO LPS in Bonferroni post hoc comparisons. D) Quantification of CD3+ T-cells, Ly6C^{low} monocytes, and Ly6C^{hi} monocytes in the brain as percentage of total CD45+ in WT and TRIFKO brains treated with either ICV LPS or saline. * = $p<0.05$, for comparing saline-treated to LPS-treated animals within the same genotype in Bonferroni post hoc comparisons. N = 4/group.

3.6 Mice lacking TRIF have attenuated cancer cachexia

TRIFKO mice inoculated orthotopically with KPC PDAC cells experienced attenuated anorexia compared to WT mice with PDAC (Fig. 5A and B). Furthermore, TRIFKO tumor mice showed attenuated voluntary locomotor activity (LMA) compared to WT tumor mice, as defined by dark cycle home cage activity (560, 561) (Fig. 5C). While Two-way ANOVA analysis of dark cycle LMA days 3-10 (time from which cachexia response was initiated) revealed a significant group effect ($F_{3, 128} = 45.75$, $p<0.001$), no post hoc comparisons between the WT tumor and TRIFKO tumor group were

significant. However, when Three-way ANOVA analysis was performed (analyzing the effects of tumor status, genotype, and time), the interaction between genotype and tumor status was significant (F = 4.35, df 1,128, p = 0.035). WT tumor-bearing mice showed significantly decreased gastrocnemius mass compared to WT sham-operated mice (Two-way ANOVA treatment effect F1,16 = 5.064, p = 0.039, WT sham vs. WT tumor p = 0.022 in post hoc analysis) while TRIFKO tumor-bearing mice did not show decreased gastrocnemius mass compared to TRIFKO sham-operated mice (p>0.99) (Fig. 5D). These effects on muscle catabolism were further evidenced by the fact that the E3 ubiquitin-ligase system genes *Mafbx* and *Murf1* were upregulated in WT tumor animals compared to WT sham animals (treatment effect p<0.001 for all three, post hoc analysis all at least p<0.01), but not significantly upregulated in TRIFKO tumor-bearing animals (interaction effect at least p<0.05)(Fig. 5E)**.** The same was true for *Foxo1*, a key transcription factor for muscle catabolism (562). In addition, although *Ccl2* was significantly upregulated in the hypothalamus of WT tumor animals compared to sham animals (treatment effect p<0.01), it was not in TRIFKO tumor-bearing animals (interaction effect p<0.05). Alternatively, compared to WT tumor-bearing animals, *Il1β* was equally upregulated in the hypothalami of TRIFKO tumor-bearing animals (treatment effect p<0.05, but interaction effect p>0.05), and *Tnf*, *Il6*, *Cd80*, *Cxcl1*, *Cxcl2*, and *Cxcl10* were not upregulated in the hypothalami WT or TRIFKO tumor-bearing animals. Lastly, although *Ccl5* was less upregulated in the hypothalami of TRIFKO tumor-bearing animals compared to WT tumor-bearing animals, this relationship was not significant (interaction effect F1,16 = 1.339, p>0.05)(Fig. 5F).
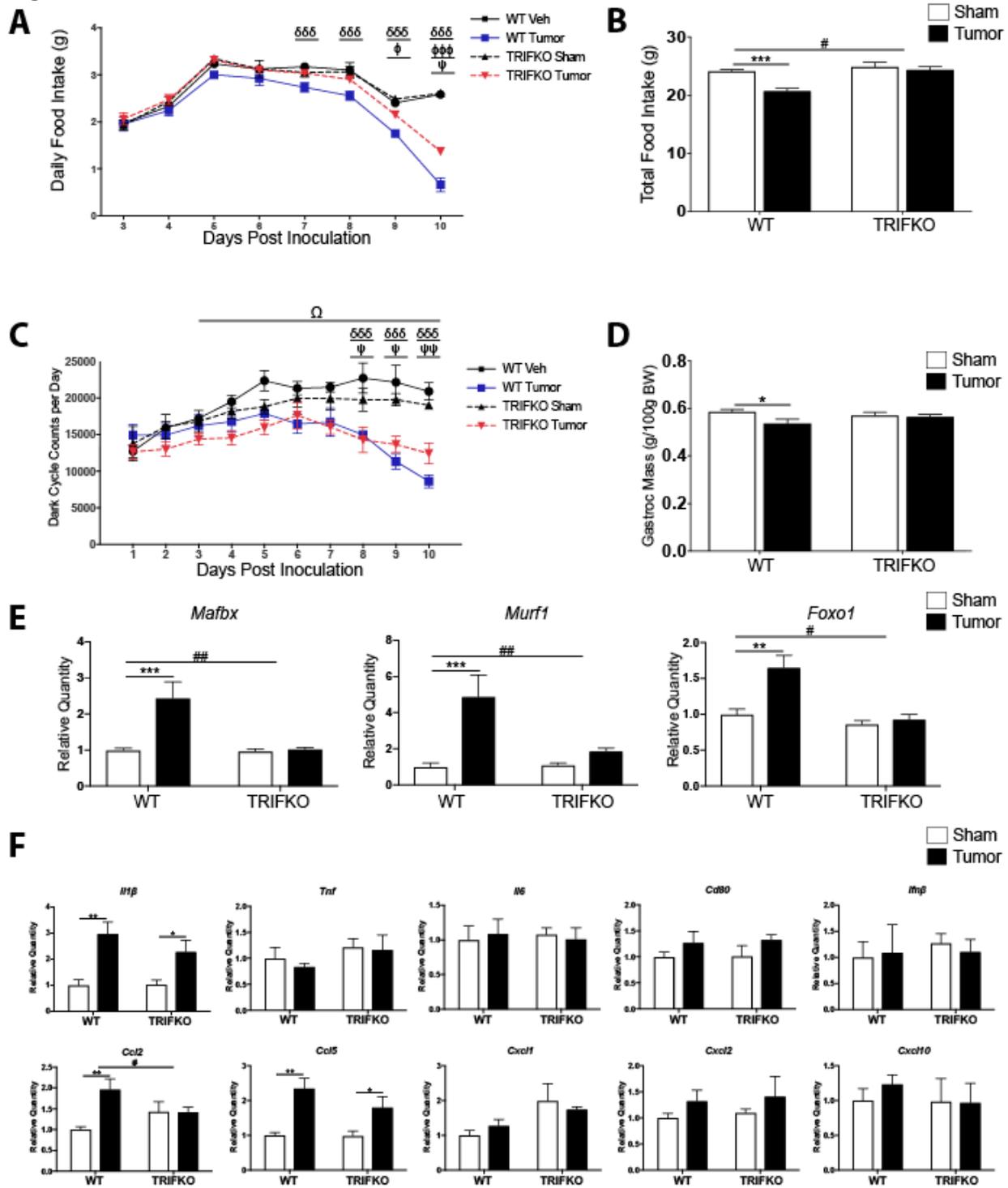
Figure 5

Figure 5: TRIFKO mice have attenuated cancer cachexia. A) Daily food intake after

a single orthotopic inoculation of 3e^6 KPC tumor cells. δδδ = p<0.001 for WT sham vs.

WT tumor in Bonferroni post hoc comparisons. Φ = p<0.05, $\Phi\Phi\Phi$ = p<0.001 for WT tumor vs. TRIFKO tumor in Bonferroni post hoc comparisons. Ψ = p<0.05 for TRIFKO sham vs. TRIFKO tumor in Bonferroni post hoc comparisons. B) Cumulative food intake 10 days post inoculation. *** = p<0.001 for WT sham vs. WT tumor in Bonferroni post hoc comparisons. # = p<0.05 for interaction in Two-way ANOVA. C) Movement quantification after inoculation with KPC tumor cells. Movement quantified using a Minimitter system with e-mitter implanted subcutaneously in between shoulder blades. $\delta\delta\delta$ = p<0.001 for WT sham vs. WT tumor in Bonferroni post hoc comparisons. Ψ = p<0.05, $\Psi\Psi$ = p<0.01 for TRIFKO sham vs. TRIFKO tumor in Bonferroni post hoc comparisons. Ω = p<0.05 for Three-way ANOVA interaction effect between genotype and treatment for days 3-10 post inoculation. D) Muscle catabolism determined by gastrocnemius mass. Mass of dissected left and right gastrocnemius was averaged and then divided by initial body weight for normalization. * = p<0.05 for WT sham vs. WT tumor in Bonferroni post hoc comparisons. E) qRT-PCR analysis of muscle catabolism genes in gastrocnemius. Expression level for all groups was normalized to WT sham. ** = p<0.01, *** = p<0.001 for WT sham vs. WT tumor in Bonferroni post hoc comparisons. # = p<0.05, ## = p<0.01 for interaction effect in Two-way ANOVA. F) Expression of inflammatory cytokine genes in the hypothalamus 10 days after orthotopic inoculation with KPC tumor cells. All data are analyzed from ΔCt values and normalized to WT sham group. * = p<0.05, ** = p<0.01, for WT sham vs. WT tumor in Bonferroni post hoc comparisons. # = p<0.05, for interaction effect in Two-way ANOVA. N = 5/group for all experiments. Data are representative from 3 independent experiments.

4. Discussion

We investigated the role of TRIF in acute sickness behavior after LPS exposure and in a model of pancreatic cancer cachexia. These studies demonstrated that TRIFKO mice experienced attenuated sickness behavior after peripheral or central LPS exposure. Furthermore, TRIFKO mice experienced attenuated cachexia during PDAC, including decreased anorexia, voluntary LMA, muscle catabolism, and hypothalamic inflammation relative to WT counterparts. These results indicate that TRIF is an important mediator of inflammation-driven sickness behavior, and should be considered during the development of anti-inflammatory therapies for cachexia.

Several studies investigated the role of MyD88 in sickness behavior (133, 563, 564), yet evidence suggests that MyD88-independent signaling pathways are important in CNS immune activation (559, 565). We found that MyD88KO mice still experienced hypothalamic inflammation. It is important to note that while many inflammatory cytokine and chemokine genes were upregulated in LPS-treated MyD88KO mice compared to saline-treated MyD88KO mice, the expression of genes in the LPS-treated MyD88KO mice was much lower than that in LPS-treated WT animals. This shows that MyD88 is a key mediator of LPS-induced neuroinflammation, but there are other mediators important in inflammatory gene expression.

While the role of TRIF signaling in acute sickness behavior during viral infection was investigated previously (133, 566, 567), these studies focused on the effects of a single injection of Poly I:C, a viral double-stranded RNA mimetic, on acute sickness behavior and neuroinflammation. Poly I:C is a known TLR3 agonist and is a model of acute viral illness. Alternatively, this is the first study to investigate the role of TRIF in

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acute sickness behavior after LPS exposure and during cachexia. After systemic challenge with LPS, TRIFKO mice experienced attenuated anorexia and weight loss compared to WT mice. These results also coincided with an attenuated increase in serum corticosterone, implicating TRIF as a key player in stress response. Furthermore, LPS-treated TRIFKO mice showed attenuated hypothalamic inflammation compared to WT mice. Interestingly, we found that *Ifnβ*, a key transcript regulated by TRIF signaling, was not induced by LPS exposure or cancer in WT or TRIFKO mice. To our knowledge, this is the first report of *Ifnβ* gene expression in the hypothalamus after systemic LPS exposure. It is possible *Ifn*β was induced earlier than 6 hrs after LPS exposure, since it was previously shown that *Ifn*β mRNA is upregulated in the hippocampus 2 hrs after 100 μg/kg IP LPS (568). Future studies should investigate the expression of type I interferons in the hypothalamus at different time points after LPS exposure.

In addition to attenuated acute sickness behavior after systemic LPS exposure, TRIFKO mice experienced attenuated anorexia and weight loss after ICV LPS administration. Interestingly, TRIFKO mice showed similar weight loss 12 hours after LPS administration, yet recovered more rapidly than WT mice, reaching baseline body weight 36 hours after injection. While it has been previously reported that TLR4 is important in neuroinflammation and acute sickness behavior after IP LPS exposure (569), the kinetics of different signaling pathways linked to TLR4 are not known. Our results suggest that in the CNS, MyD88 may drive initial sickness response after TLR4 activation, whereas TRIF signaling may be involved in maintaining inflammation and subsequent sickness response. It should be noted that a repeated measures three-way ANOVA would provide valuable information on the interaction between time, genotype,

and treatment status. Unfortunately, our experiments were not sufficiently powered for this analysis. Future studies should investigate the kinetics of TRIF-dependent acute sickness behavior in detail.

In the brain, microglia express TRIF at basal levels, and this expression is enhanced by various CNS insults (549, 570). Review of the published online database (https://web.stanford.edu/group/barres_lab/brain_rnaseq.html) confirms that the basal expression of TRIF (*Ticam1*) is predominantly found in microglia (132). We found that, unlike in WT mice, microglia in TRIFKO mice did not show an increase in Iba-1 intensity in the ME or an increase in Iba-1+ cell size in the ARC 12 hrs after ICV LPS administration. While additional studies are needed to characterize the functional changes in TRIFKO microglia after LPS exposure, these results are in agreement with previous studies showing that TRIF expression in microglia is required for normal inflammatory activation and phagocytosis in response to neuronal injury (549, 559). Furthermore, in a murine model of intracerebral hemorrhage, TRIFKO mice showed attenuated neurologic disability and neuroinflammation. In addition, TLR2 activation in hypothalamic microglia was shown to generate sickness responses (162). TRIF is now known to be linked to TLR2 signaling (571, 572). Therefore, our data, in addition to previous findings, suggest TRIF is important in microglial activation during states of inflammation, which is important in driving subsequent functional and behavioral response.

We found that TRIFKO mice experienced attenuated hypothalamic inflammation after systemic LPS exposure. Interestingly, amongst the differentially regulated transcripts between WT and TRIFKO animals, there was a predominance of chemokine

mRNAs (*Ccl2*, *Ccl5*, *Cxcl1*, *Cxcl10*). Previous studies showed that acute LPS exposure results in peripheral immune cell recruitment to the brain (232) and that infiltrating immune cells in the brain drive sickness behavior in states of chronic inflammation (178). Based on these data, we investigated whether TRIFKO mice had decreased immune cell infiltration into the brain after ICV LPS exposure and found that TRIF was required for neutrophil recruitment. Interestingly, we did see a nonsignificant increase in Ly6Chi monocytes. While it is difficult to speculate on trends, it is possible there was a compensatory increase in the cells as a result of TRIF deletion causing an altered inflammatory reaction. While TRIF is known to be important for neutrophil recruitment to the lungs (573), this is the first study to implicate TRIF in neutrophil recruitment to the brain. This presents a novel mechanism that can be applied to several pathologies, including CNS infection, cancer, and stroke. Furthermore, while neutrophils may appear after the onset of initial sickness behaviors in the setting of acute illness, no studies have investigated whether neutrophils are important in sickness behavior or cachexia. In addition, it is important to note that while our flow cytometry results cannot distinguish if the CNS neutrophils have translocated into the brain parenchyma, several lines of evidence demonstrate that adherent intravascular neutrophils contribute to inflammation and pathology in the brain (574, 575). Interestingly, brain-infiltrating neutrophils in LPS-treated TRIFKO mice showed a nonsignificant increase in Ly6G expression compared to brain-infiltrating neutrophils in LPS-treated WT mice, sometimes so much so that the Ly6G expression exceeded the pre-defined "maximum" fluorescent intensity. This suggests that brain-infiltrating neutrophils in TRIFKO mice may have a different phenotype than those in WT mice. More advanced flow cytometry studies (intracellular

staining for cytokines and enzymes) and histology studies are needed to further investigate this observation.

When inflammation is maintained, acute sickness behavior transforms into cachexia, a maladaptive condition associated with increased mortality and decreased quality of life during numerous chronic diseases (539-541). While inflammation is critical for cachexia, mechanisms of inflammatory signaling important for this syndrome remain unclear. We found that in a mouse model of PDAC-associated cachexia, TRIFKO mice experienced attenuated anorexia, voluntary LMA, muscle catabolism, and hypothalamic inflammation compared to WT mice. While our results were consistent across various behavioral and molecular measures of cachexia, it is important to note that the effects of *Ticam1* deletion were modest. It is important to note that differences in dark cycle home cage LMA between WT tumor and TRIFKO tumor were significant only when analysis was expanded to Three-way ANOVA, a testament to the minor effects of TRIF on this measure of cachexia. These results suggest that other inflammatory signaling pathways are important in cachexia. Rudd et al. previously reported that MyD88KO mice were resistant to anorexia in a methylcholanthrene-induced sarcoma tumor model (130). However, this was a short report and their analysis of cachexia was limited to food intake and carcass weight.

The main limitation of the present study is the lack of cell specificity in global TRIFKO experiments. Future studies are needed to identify the critical cell type involved in TRIF-mediated sickness behavior and cachexia. Furthermore, we only assessed muscle catabolism only in the gastrocnemius. While gastrocnemius may not be representative of all skeletal muscle, we previously reported that other muscle groups

(tibialis anterior, soleus, etc.) showed similar catabolic changes during PDAC cachexia (122). In addition, we only used a single measure of stress response (circulating corticosterone levels 4 hrs after LPS exposure). While a single test is not sufficient to fully define the role of TRIF in stress response, previous studies have shown 4 hr circulating corticosterone levels as a robust measure of acute stress response (576, 577). In addition, we report results from multiple experiments using different doses ICV LPS administration. We chose a dose sufficient to induce sickness response when injected centrally, but not when injected peripherally (50 ng), and the smallest single dose required to induce neutrophil recruitment to the brain (500 ng, data not shown). While whether or not LPS crosses the blood brain barrier (BBB) remains controversial (578), we believe this route of administration is sufficient for the purpose of this study for two reasons. First, the MBH contains a dramatically attenuated BBB, allowing circulating molecules to access the parenchyma in the region (579, 580). Second, there are several ways that peripherally administered LPS induces neuroinflammation (acute phase response proteins, platelet mobilization, peripheral immune cell-derived cytokines, vagus nerve activation, etc.) (553, 581). Therefore, we chose to make many of our observations with low-dose LPS delivered centrally to minimize confounders. Another limitation is the fact that we performed analysis of cachexia using only one mouse model of cancer cachexia. Caution is warranted when applying our results to other types of cachexia (heart failure, cirrhosis, untreated HIV, other types of cancer, etc.). However, this model is extensively characterized (122), and recapitulates all of the cardinal features of cachexia seen in humans. Furthermore, it avoids many of the shortcomings in other mouse models of cachexia, including: multiple clones with

variable cachexia (582), cachexia driven by only a single cytokine (583), and requiring advanced surgical techniques to induce cachexia (584). Nevertheless, future studies should assess the effects of *Ticam1* deletion in other models of cachexia, as well as the tumor immune response in TRIFKO mice.

In conclusion, we report that TRIF is important in acute sickness behavior and cachexia. These results show that TRIF-dependent mechanisms should be considered when developing therapeutic targets for cachexia. Future studies are needed to identify the important cell types involved in TRIF signaling during acute illness response and cachexia.

Competing Interests

The authors have no competing interests to report.

Acknowledgements

Supplemental Tables and Figures

Figure S1
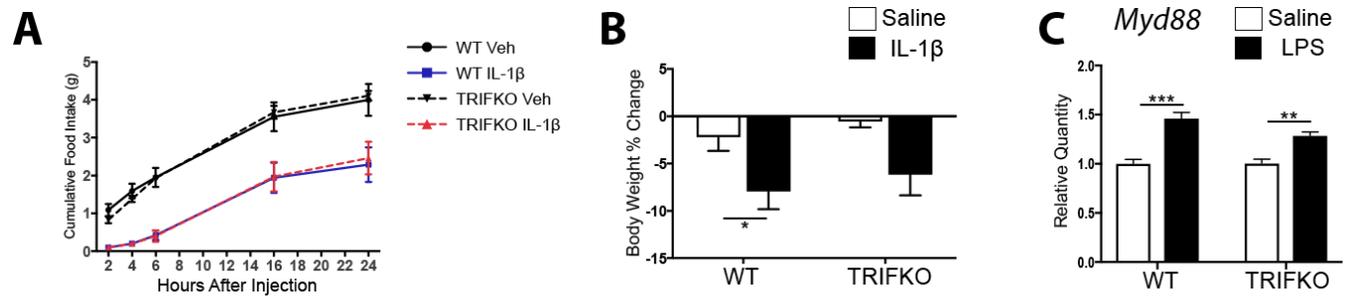
Figure S1: MyD88 signaling is intact in TRIFKO mice. A) Cumulative food intake after 10 ng ICV IL-1β in artificial CSF or vehicle (artificial CSF only) treatment. B) Body weight change from initial after 10 ng ICV IL-1β in artificial CSF or vehicle (artificial CSF only) treatment. N=6-8/group. C) qRT-PCR for *Myd88* gene expression in WT and TRIFKO mice. LPS = 8 hrs after 250 μg/kg IP LPS treatment. N = 3-4/group. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ for two-way ANOVA Bonferroni post hoc testing.

Figure S2
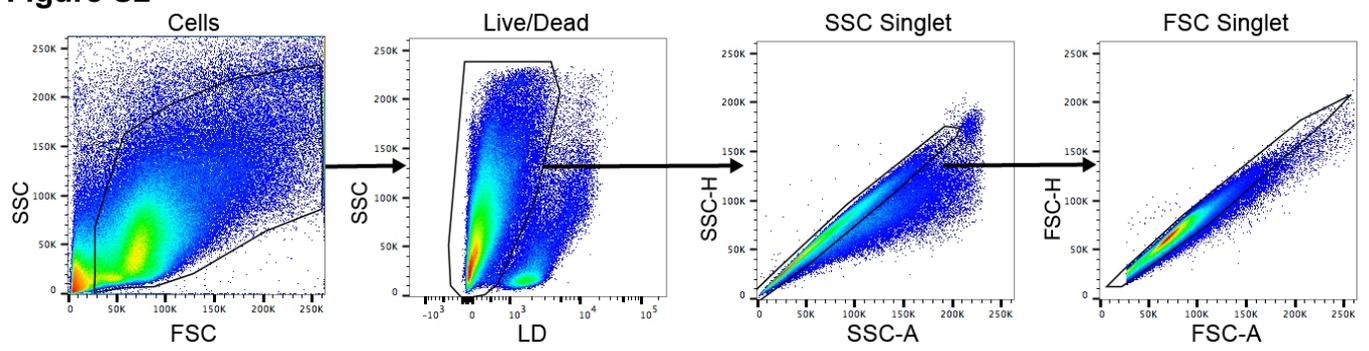
Figure S2: Flow cytometry gating strategy. Representative plots from FlowJo V10 software. SSC = side scatter. FSC = forward scatter. A = area. H = height. LD = Live/Dead.

Figure S3
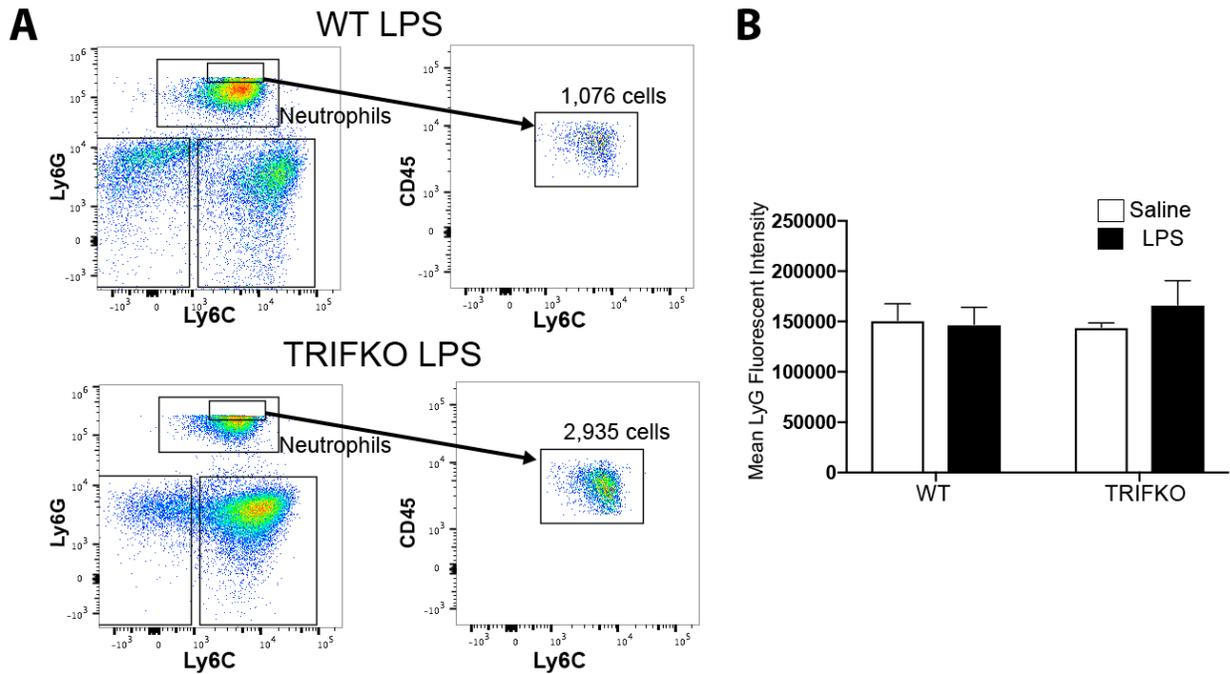
Figure S3: Neutrophil gating strategy. A) Gates drawn within neutrophil gates at edge of maximum Ly6G (PE/Cy7) fluorescent intensity showing that any cell with an intensity beyond $10^{5.5}$ is displayed as having an intensity of $10^{5.5}$, yet is still included within neutrophil gate, as evidenced by Ly6G and CD45 expression visualization. B) Ly6G mean fluorescent intensity in WT and TRIFKO brain neutrophils. Note slightly increased Ly6G intensity in TRIFKO LPS group (treatment, genotype, and interaction $p > 0.05$ in Two way ANOVA).

Chapter 4: A Distinct Neutrophil Population Invades the Central Nervous System to Drive Cachexia During Pancreatic Cancer.

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Title: "A distinct neutrophil population invades the central nervous system to drive cachexia during pancreatic cancer"

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Summary

Cachexia, a wasting syndrome that frequently occurs during cancer, is associated with brain inflammation, but cellular mediators are unknown. The authors showed that during pancreatic cancer neutrophils infiltrate the brain, via a novel process dependent on CCR2, where they drive cachexia.

Graphical Abstract
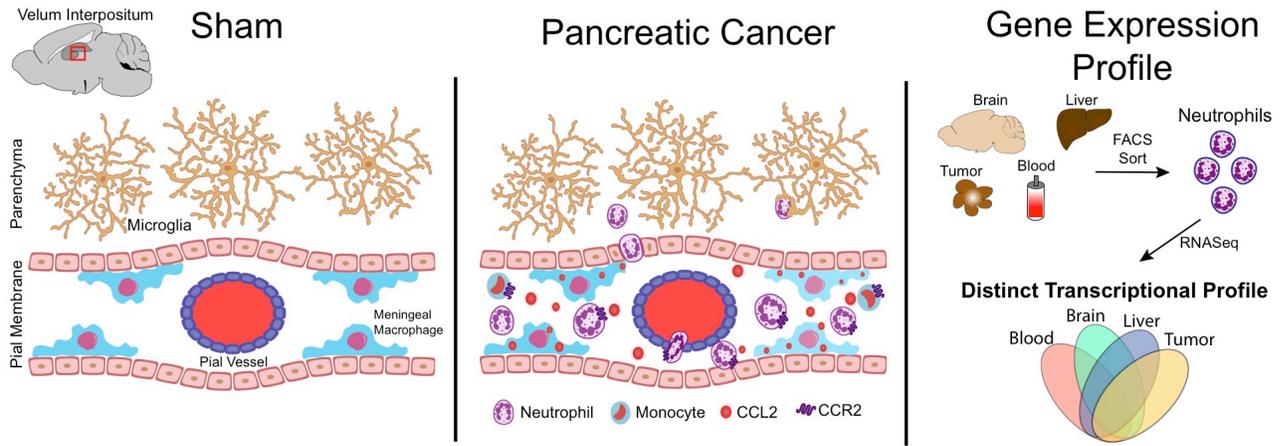
Abstract

Cachexia is a devastating syndrome consisting of anorexia, muscle catabolism, and fatigue. Inflammation in the central nervous system (CNS) can cause cachexia, yet cellular sources of neuroinflammation during this syndrome are not known. In a mouse model of pancreatic ductal adenocarcinoma (PDAC)-associated cachexia, we observed robust myeloid cell infiltration into the brain. Infiltrating immune cells were predominately neutrophils, accumulating at a unique CNS entry portal, where they expressed CCR2, an atypical granulocyte receptor. CCR2 knockout (CCR2KO) mice had significantly decreased infiltrating neutrophils in the brain and attenuated cachexia compared to WT mice, without any changes in neutrophils in other organs. Intracerebroventricular blockade of the purinergic receptor P2RX7 during PDAC abolished neutrophil recruitment to the brain and attenuated cachexia. Lastly, RNAseq analysis revealed a distinct transcriptional profile in brain-infiltrating neutrophils compared to those in the blood, liver, and tumor. Our data provide a novel function for the CCR2/CCL2 axis in recruiting a transcriptionally distinct neutrophil population to the brain that drives cachexia.

1. Introduction

Cachexia is a devastating state of malnutrition characterized by decreased appetite, fatigue, adipose tissue loss, and muscle catabolism (30). This syndrome accompanies many chronic diseases, including cancer, chronic obstructive pulmonary disease, rheumatoid arthritis, and congestive heart failure (585). There are currently no effective treatments for cachexia, and mechanisms remain controversial. The one unifying feature of all diseases with associated cachexia is inflammation. While inflammation occurs in multiple organ systems, inflammatory molecules (*e.g.*, lipopolysaccharide, cytokines) can cause dysfunction of the appetite-, weight-, and activity-regulating regions in the central nervous system (CNS), resulting in signs and symptoms nearly identical to those in cachexia (121, 124, 457). Moreover, cytokines and chemokines are produced in these same regions during cachexia-associated diseases such as cancer (122), parasitic infection(586), and inflammatory liver disease (178, 179). Our lab and others previously showed that disrupting inflammatory signaling by deleting either MyD88 or TRIF attenuates anorexia, muscle catabolism, fatigue, and neuroinflammation during cancer-associated cachexia (123, 587).

The mechanisms by which inflammation generated in the periphery (*e.g.*, at the site of a malignancy) is translated into inflammation in the brain, and how this is subsequently translated into cachexia, are still not known. Circulating immune cells present an intriguing cellular candidate, as they are thought to infiltrate and interact with the brain during various states of inflammation (135), yet have not been investigated as potential mediators of cachexia. Therefore, we characterized the identity, properties, and function of infiltrating immune cells in the brain during cachexia using a syngeneic,

immunocompetent, mouse model of pancreatic ductal adenocarcinoma (PDAC), a potently cachexigenic malignancy (122). We found that circulating myeloid cells, primarily neutrophils, were recruited to the CNS early in PDAC-associated cachexia, infiltrating throughout the brain parenchyma and accumulating in the meninges near regions important for appetite, behavior, and body composition regulation. We then show a novel role for the CCR2-CCL2 axis (typically considered a monocyte chemotaxis pathway) in recruiting neutrophils specifically to the brain, rather than the liver or tumor. We then blocked purinergic receptor P2RX7 signaling specifically on brain macrophages during PDAC via intracerebroventricular (ICV) injection of oxidized ATP (oATP), which prevented circulating myeloid cell recruitment to the brain and attenuated cachexia. Lastly, we showed that during PDAC, brain-infiltrating neutrophils have a transcriptional profile that is distinct from that of circulating, liver-infiltrating, and tumor-infiltrating neutrophils. Taken together, these results reveal a novel mechanism for neutrophil recruitment to the brain, in which a transcriptionally distinct population is recruited via an atypical neutrophil chemotactic factor, in a manner distinct from that in the periphery.

2. Materials and Methods

2.1 Animals

Male and female 20–25g WT C57BL/6J (stock no. 000664), Ly5.1-EGFP (stock no. 00657), CCL2^{mCherry} (stock no. 016849), and CCR2KO (stock no. 004999) were purchased at Jackson Laboratories. Animals were aged between 7 and 12 weeks at the time of study and maintained at 27°C on a normal 12:12 hr light/dark cycle and provided *ad libitum* access to water and food. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Oregon Health & Science University.

2.2 KPC Cancer Cachexia Model

Our lab generated a mouse model of PDAC-associated cachexia by a single injection of murine-derived KPC PDAC cells (originally provided by Dr. Elizabeth Jaffee from Johns Hopkins) (122). These cells are derived from tumors in C57BL/6 mice heterozygous for oncogenic KRAS^{G12D} and point mutant TP53^{R172H} with expression targeted to the pancreas via the PDX-1-Cre driver (554). Cells were maintained in RPMI supplemented with 10% heat-inactivated FBS, and 50 U/mL penicillin/streptomycin (Gibco, Thermofisher), in incubators maintained at 37°C and 5% CO_2. In the week prior to tumor implantation, animals were transitioned to individual housing to acclimate to experimental conditions. Animal food intake and body weight were measured once daily. Sham-operated animals received PBS in the same volume. Bedding was sifted daily to account for food spillage not captured by cagetop food intake measurement.

Animals were euthanized between 8 and 11 days post inoculation, when food intake was consistently decreased and locomotor activity was visibly reduced, yet signs of end-stage disease (ascites, unkempt fur, hypotheremia, etc.) were not present (122).

2.3 Generation of Ly5.1-EGFP Chimera Mice

WT C57BL/6J male mice aged 8-10 weeks were injected IP with the alkylating agent treosulfan (Ovastat®, a generous gift from Joachim Baumgart at Medac GmbH, Germany) at a dose of 1500 mg/kg/day for 3 consecutive days prior to the day of bone marrow transplant (BMT). 24 hrs after the third treosulfan injection, a Ly5.1-EGFP male or female donor mouse aged between 2-6 months was euthanized and femurs, tibias, humeri, and radii were dissected. After muscle and connective tissue were removed, marrow cells were harvested by flushing the marrow cavity of dissected bones using a 25-gauge needle with Iscove's modified Dulbecco's medium supplemented with 10% FBS. The harvested cells were treated with RBC lysis buffer, filtered with a 70 µm cell strainer, and counted. 3-4 × 106 cells in 200 μL HBSS were transplanted immediately into each recipient mouse via tail vein injection. To prevent infection during an immunocompromised period, recipient mice received amoxicillin dissolved in their drinking water (150 mg/L) for 2 weeks starting on the first day of treosulfan injection. GFP BMT mice were given at least 5 weeks for marrow reconstitution and recovery. Percent chimerism in each GFP BMT mouse was determined by flow cytometry analysis of circulating leukocytes.

2.4 Behavioral Analysis

Behavioral and cognitive tests were performed on days 7-9 d.p.i. The open field test was conducted on days 7 and 8 post-inoculation, and the object recognition test was performed on days 8 and 9 post-inoculation. For all behavioral analyses, observers were blinded to group (tumor vs. sham).

Open field Testing. Exploratory and anxiety-like behaviors were assessed using the open field test on two subsequent days. The open field consisted of a brightly lit square arena (L 40.6 × W 40.6 × H 40.6 cm). The light intensity in the center of the open field was 100 lux. Mice were allowed to explore for 10 min in each trial. Behavioral performance was tracked and analyzed using an automated video system (Ethovision 7.0 XT, Noldus). Exploratory behavior was analyzed and included total distance moved and time spent in the center (20 × 20 cm) of the open field.

Novel Object Recognition. Mice were habituated to the open field arena over two days as described above on two subsequent days. On the third day, mice were exposed to the arena containing two identical objects (small orange hexagonal prisms) placed 15 cm from the adjacent walls and 10 cm apart for 15 min. On day four, one of the identical objects ("familiar") was replaced with a novel object (small green triangular prism) of similar dimensions and mice were again allowed to explore for 15 min. During both the open field and novel object recognition tests, mice were placed into the center of the arena. Clear visuospatial orientation to the object, within 2 cm proximity, as well as physical interaction with the object was coded as exploratory behavior, and the percent time spent exploring the novel versus the familiar object was calculated. Three

tumor animals were excluded from analysis because of complete lack of exploratory behavior.

2.5 Intracerebroventricular Cannulation and Injections

Mice were anesthetized under isoflurane and placed on a stereotactic alignment instrument (Kopf Instruments). 26-gauge lateral ventricle cannulas were placed at 1.0 mm X, −0.5 mm Y, and −2.25 mm Z relative to bregma. Mice were given one week for recovery after cannula placement. Injections were given in 2 µl total volume. Oxidized ATP was dissolved in aCSF and injected at a concentration of 250 ng/µL over 5 min while mice were anesthetized under isoflurane.

2.6 Immunofluorescence Immunohistochemistry

Mice were anesthetized using a ketamine/xylazine/acetapromide cocktail and sacrificed by transcardial perfusion fixation with 15 mL ice cold 0.01 M PBS followed by 25 mL 4% paraformaldehyde (PFA) in 0.01 M PBS. Brains were post-fixed in 4% PFA overnight at 4°C and cryoprotected in 20% sucrose for 24 hrs at 4°C before being stored at −80°C until used for immunohistochemistry. Immunofluorescence immunohistochemistry was performed as described below. Free-floating sections were cut at 30 µm from perfused brains using a Leica sliding microtome. Sections were incubated for 30 min at room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS and 0.3% Triton X-100). After the initial blocking step, sections were incubated in primary antibody (listed below) in blocking reagent for 24 hrs at 4°C, followed by incubation in secondary antibody (also listed below) for 2 hrs at room temperature. Between each stage,

sections were washed thoroughly with 0.01 M PBS. Sections were mounted onto gelatin-coated slides and coverslipped using Prolong Gold antifade media with DAPI (Thermofisher).

The following primary anti-mouse antibodies were used, with company, clone, host species, and concentration indicated in parentheses: CD11b (eBioscience, rat, M1/70, 1:1000), CD45 (BD, rat, 30-F11, 1:1000), myeloperoxidase (R&D, goat, polyclonal, 1:1000), Ly6G (Biolegend, 1A8, rat, 1:250), Iba-1 (Wako, Rabbit, NCNP24, 1:1000), CD206 (Bio-rad, rat, MR5D3, 1:1000), ER-TR7 (Abcam, rat, ER-TR7, 1:1000), and citrillunated histone H3 (Abcam, rat, polyclonal, 1:1000). We also used a chicken anti-mCherry antibody (Novus Biologicals, polyclonal, 1:20,000), to amplify mCherry signal in sections from CCL2fl/fl mice and a rabbit anti-RFP antibody (Abcam, polyclonal, 1:1000) to amplify RFP signal in sections from CCR2RFP/WT mice.

The following secondary antibodies were used, all derived from donkey and purchased from Invitrogen, with dilution in parentheses: anti-goat AF488 (1:500), anti-rabbit AF555 (1:1000), anti-rat AF555 (1:1000), anti-rat AF633 (1:500), and anti-chicken AF555 (1:1000).

2.7 Image acquisition and analysis

All images were acquired using a Nikon confocal microscope. Cell quantification was performed on 20X images using the Fiji Cell Counter plugin by a blinded researcher. CD45+ cells were defined as CD45 bright globoid cells, and neutrophils were defined as CD45+ MPO+ cells. The velum interpositum (VI) was defined as the layer of meninges (identified by appearance of staining background) between the hippocampus and

thalamus, from bregma -1.7 to -2.6 mm. At least 8 VI images were quantified from each animal. The median eminence was defined as the base of the mediobasal hypothalamus (far ventral part of the brain), adjacent to the third ventricle from bregma -1.95 to -2.5 mm. Four ME images were quantified from each animal. The area postrema was defined as the region in from bregma -7.2 to -7.75 mm. Four area postrema images were quantified from each animal.

2.8 Microglia morphology analysis

Microglia activation in the hippocampus was quantified using Fiji (ImageJ, NIH). Five images of the dentate gyrus were acquired from each animal. Images were 2048 x 2048 pixels, with a pixel size of 0.315 μm. Images were uploaded to Fiji by a blinded reviewer (KGB) and converted to 8-bit greyscale images. After thresholding, microglia were identified using the "analyze particle" function, which measured mean Iba-1 fluorescent intensity per cell, cell area, and percent area covered by Iba-1 staining.

2.9 *In situ* hybridization

At 10 d.p.i., mice were euthanized with CO_2 and brains were removed then frozen on dry ice. 20 μm coronal sections were cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific). Sections were collected in a 1:6 series from the diagonal band of Broca (bregma 0.50 mm) caudally through the mammillary bodies (bregma 5.00 mm). 0.15 pmol/ml of an antisense 33P-labeled mouse Ccl2 riboprobe (corresponding to bases 38-447 of mouse Ccl2; GenBank accession no. NM_011333.3) was denatured, dissolved in hybridization buffer along with 1.7 mg/ ml tRNA, and

applied to slides. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55°C. The following day, slides were treated with RNase A and washed under conditions of increasing stringency. Slides were dipped in 100% ethanol, air dried, and then dipped in NTB-2 liquid emulsion (Kodak). Slides were developed 4 d later and cover slipped.

2.10 Quantitative Real-Time PCR

Prior to tissue extraction, mice were euthanized with a lethal dose of a ketamine/xylazine/acetapromide and sacrificed. Hippocampal blocks and gastrocnemii were dissected, snap frozen, and stored in −80 °C until analysis. RNA was extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was transcribed using TaqMan reverse transcription reagents and random hexamers according to the manufacturer's instructions. PCR reactions were run on an ABI 7300 (Applied Biosystems), using TaqMan universal PCR master mix with the following TaqMan mouse gene expression assays: 18s (Mm04277571_s1), Ccl2 (Mm99999056_m1), Ccl3 (Mm00441259_g1), Ccl5 (Mm01302427_m1), Cxcl1 (Mm04207460_m1), Cxcl2 (Mm00436450_m1), Cxcl5 (Mm00436451_g1), Cxcl9 (Mm00434946_m1), Cxcl10 (Mm00445235_m1), Gapdh (Mm99999915_g1), Mafbx (Mm00499518_m1), Murf1 (Mm01185221_m1), and Foxo1 (Mm00490672_m1). Relative expression was calculated using the $\Delta\Delta$Ct method and normalized to WT vehicle treated or sham control. Statistical analysis was performed on the normally distributed ΔCt values.

2.11 Flow cytometry

Mice were anesthetized using a ketamine/xylazine/acetapromide cocktail and perfused with 15 mL ice cold 0.01 M PBS to remove circulating leukocytes. If circulating leukocytes were analyzed, blood was drawn prior to perfusion via cardiac puncture using a 25-gauge needle, then placed in an EDTA coated tube. After perfusion, organs were extracted and immune cells were isolated using the following protocols:

Brain. Brains were minced in a digestion solution containing 1 mg/mL type II collagenase (Sigma) and 1% DNAse (Sigma) in RPMI, then placed in a 37°C incubator for 45 min. After digestion, myelin was removed via using 30% percoll in RPMI. Isolated cells were washed with RPMI, incubated in Fc block for 5 min, then incubated in 100 µL of PBS containing antibodies for 30 min at 4°C. Cells were then washed once with RPMI.

Liver. Livers were pushed through a 70 µm nylon strainer, then washed once with RPMI. The resulting suspension was resuspended in a 40 mL digestion solution containing 1 mg/mL type II collagenase (Sigma) and 1% DNAse (Sigma) in RPMI, then placed in a 37°C incubator for 1 hr. After digestion, the suspension was placed on ice for 5 min, then the top 35 mL was discarded. The remaining 5 mL was washed in RPMI, resuspended in 10 mL 35% percoll to remove debris, then treated with RBC lysis buffer. The resulting cell suspension was washed with RPMI, then cells were incubated in 100 µL of PBS containing antibodies for 30 min, then washed with RPMI.

Tumor. A 0.4-0.5 g piece of pancreatic tumor was removed, minced in a digestion solution containing 1 mg/mL type II collagenase (Sigma) and 1% DNAse (Sigma) in RPMI, then placed in a 37°C incubator for 1 hr. After digestion, cells were washed with RPMI, then incubated in 100 µL of PBS containing antibodies for 30 min at 4°C. Cells were then washed once with RPMI.

Blood. 200 µL of blood was drawn via cardiac puncture with a 25-gauge needle. Red blood cells were then lysed with 1X RBC lysis buffer. The resulting cell suspension was washed with RPMI, then cells were incubated in 100 µL of PBS containing antibodies for 30 min at 4°C, then washed with RPMI.

Gating Strategy. Cells were gated on LD, SSC singlet, and FSC singlet. Immune cells were defined as CD45+ cells. In the brain, microglia were defined as CD45midCD11b+. Leukocytes were identified as either myeloid cells (CD45highCD11b+ in the brain, CD45+CD11b+ in all other tissues) or lymphocytes (CD45highCD11b- in the brain, CD45+CD11b- in all other tissues). From myeloid cells Ly6Clow monocytes (Ly6ClowLy6G-), Ly6Chigh monocytes (Ly6ChighLy6G-), and neutrophils (Ly6CmidLy6G+) were identified. From lymphocytes, CD3+ cells were identified as T-cells, and further phenotyped as CD4+ or CD8+ T-cells. CD3- T-cells were divided into NK1.1+ NK cells or CD19+ B-cells. Flow cytometry analysis was performed on a BD Fortessa or LSRII analytic flow cytometer.

Antibodies. All antibodies were purchased from BioLegend, except for Live/Dead, which was purchased from Invitrogen (Fixable Aqua, used at 1:200 dilution). The following anti-mouse antibodies were used, with clone, fluorophore, and dilution indicated in parenthesis: CD3 (17A2, PE, 1:100), CD3 (17A2, APC/Cy7, 1:400), CD4 (RM4-5, APC, 1:100), CD8 (53-6.7, APC/Cy7, 1:800), CD11b (M1/70, APC, 1:800), CD11b (M1/70, FITC, 1:200), CD19 (6D5,BV650, 1:33), CD45 (30-F11, PerCP/Cy5.5, 1:400), CD45 (30-F11, APC/Cy7, 1:400), Ly6C (HK1.4, PerCP, 1:100), Ly6G (1A8, PE/Cy7, 1:800), NK1.1 (PK136, BV785, 1:800).

FACS sorting for RNAseq. At 10 d.p.i., mice were anesthetized and 200 µL of blood was drawn via cardiac puncture with a 25-gauge needle. Circulating leukocytes were then removed via transcardiac perfusion with PBS and brain, liver, and tumor were removed. Leukocytes were isolated from blood, brain, liver, and tumor as described above. Sorting was performed using an Influx sorter (BD) with a 100 µm nozzle. Neutrophils were defined as CD11bhighLy6Ghigh live, singlet cells, and were sorted into lysis buffer (Qiagen), then stored at -80° C.

2.12 RNA Isolation and Sequencing

RNA Isolation, sequencing, and library preparation. Total RNA was isolated from FACS-sorted CD11bhighLy6Ghigh neutrophils using an RNAeasy Plus Micro kit (Qiagen). RNA integrity was verified by a Bioanalyzer (Agilent). Sample cDNAs were prepared using the SMART-Seq v4 Ultra Low Input kit (Takara) using 250 pg of input total RNA followed by library preparation using a TruSeq DNA Nano kit (Illumina). Libraries were

verified by Tapestation (Agilent). Library concentrations were determined by real-time PCR with a StepOnePlus Real Time PCR System (Thermo Fisher) and a Kapa Library Quantification Kit (Kapa Biosystems / Roche). Libraries were sequenced with a 100 cycle single read protocol on a HiSeq 2500 (Illumina) with four libraries per lane. Fastq files were assembled using Bcl2Fastq2 (Illumina).

RNA-seq processing and analysis. Quality control checks were done using the FastQC package (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were normalized and analyzed using the Bioconductor package DESeq2 (588), which uses negative binomial generalized linear models. Only those genes that were expressed in at least one sample were included in differential expression analysis. To identify transcripts differentially expressed in brain-infiltrating neutrophils compared to neutrophils infiltrating other organs, gene expression in neutrophils isolated from brain was compared to that in neutrophils isolated from liver, tumor, and blood. In order to control for the effects of tumor on circulating neutrophils, genes that also were differentially expressed in circulating neutrophils from tumor mice compared to circulating neutrophils from sham mice were excluded from analysis. All p-values were adjusted for multiple comparisons using the Benjamani-Hochberg method (589). Differential expression was defined based on statistical significance (adjusted p-value < 0.05) and effect size (log2 fold change) ≤ or ≥ -2. Heatmaps were created using the pheatmap package from R. Gene Ontology analysis was performed using the Goseq Bioconductor R package(590). For pathway enrichment analysis, pathway annotation from the Reactome knowledgebase (591, 592) was used.

2.13 Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed with Prism 7.0 software (Graphpad Software Corp). When two groups were compared, data were analyzed with either student's t-test or Mann-Whitney U test. When more than two groups were compared, data were analyzed with either One-way (when multiple groups were compared to a single sham group) or Two-way (when there were multiple genotypes within tumor and sham groups being compared) ANOVA analysis. For single time point experiments, the two factors in ANOVA analysis were genotype or treatment. In repeated measures experiments, the two factors were group and time. Main effects of genotype, treatment, group, and/or time were first analyzed, and if one effect was significant, Bonferroni post hoc analysis was then performed. For all analyses, significance was assigned at the level of $p < 0.05$.

3. Results

3.1 Circulating myeloid cells infiltrate the brain early in PDAC cachexia

We first investigated whether circulating immune cells infiltrate the brain throughout the course of PDAC cachexia. We utilized a mouse model of PDAC cachexia, generated through a single intraperitoneal (IP) injection of C57BL/6 KRAS^{G12D} P53^{R172H} Pdx-Cre$^{+/+}$ (KPC) cells. This well-characterized model recapitulates all of the key signs and symptoms of cachexia seen in humans, including anorexia, muscle catabolism, fatigue, and loss of fat mass (122). Using 10-color flow cytometry of whole brain homogenate (Fig 1A), we characterized brain immune cells at three time points: 5 days post-inoculation (d.p.i) (before cachexia onset), 7 d.p.i. (cachexia initiation), and 10 d.p.i. (mid/late cachexia, but 2-3 days before death) (see either ref 6 or Fig 5F for typical cachexia progression in our KPC model). Compared to sham-injected animals, we observed a significant increase in CD45^{high}CD11b+ myeloid cells in the brains of animals with PDAC (Fig 1B), with an increase as a percentage of total CD45+ (all immune cells) and CD45^{high} (non-microglia leukocytes) cells occurring at 5 d.p.i. (Fig 1D and Fig S1D). Both absolute and relative number of lymphocytes (CD45^{high}CD11b-) were decreased in the brains of tumor animals compared to sham animals starting at 5 d.p.i., which was driven by a decrease in B-cells and CD4+ T-cells (Fig 1C and Fig S1B-D). There was no change in number of microglia (defined as CD45^{mid}CD11b+) throughout the course of cachexia (Fig 1C). Further phenotypic analysis of infiltrating myeloid cells revealed that by 7 d.p.i., there was an increase in relative number (as a percentage of total CD45+ and CD45^{high}) of Ly6C^{mid}Ly6G^{high} neutrophils, Ly6C^{low} myeloid cells, and Ly6C^{high} monocytes (Fig 1D, E and Fig S1D),

which correlated with cachexia onset. We observed an increase in absolute number of neutrophils, Ly6C^{high} monocytes, and Ly6C^{low} myeloid cells starting at 7 d.p.i., which became significant at either 7 (Ly6C^{high} monocytes) or 10 d.p.i. (neutrophils and Ly6C^{low} myeloid cells) (Fig 1D and E). Neutrophils were by far the most numerous invading myeloid cell type, constituting 34% percent of CD45^{high}CD11b+ cells in sham animals, and increasing to nearly 54% by 10.d.p.i. in tumor animals (Fig 1F).

To verify the identity of the CD45^{high}CD11b+ cells in the brain as invading circulating myeloid cells, we generated GFP+ bone marrow chimera mice through conditioning WT mice with treosulfan to ablate marrow, then transplanting marrow from pan-GFP mice (Ly5.1^{GFP}) (Fig 1G). This system is advantageous because, unlike other alkylating agents, treosulfan does not cross or disrupt the blood brain barrier (593). On average, mice that underwent bone marrow transplant (GFP BMT mice) exhibited 75% chimerism (Fig 1I). In agreement with results from WT marrow animals, we observed that at 10 d.p.i., thousands of GFP+ myeloid cells infiltrated the brain in tumor animals (Fig 1H). The majority of these cells were neutrophils, with a concurrent increase in Ly6C^{high} monocytes (Fig 1J-L). As we observed previously, this coincided with a decrease in brain lymphocytes (CD45+GFP+CD11b-) in tumor animals (Fig 1J). We did not observe an increase in GFP+ Ly6^{low} myeloid cells (Fig 1L), suggesting that the increase in CD45^{high}CD11b+Ly6C^{low} cells in our WT marrow PDAC mice was a result of microglia activation, rather than infiltrating monocytes.

Taken together, these data show that myeloid cells infiltrate the brain during PDAC, which correlates with the onset of cachexia, and that there is a selective neutrophil recruitment. Since the purpose of this study was to investigate infiltrating cells,

we chose to focus our subsequent analysis on myeloid cells, with an emphasis on neutrophils.
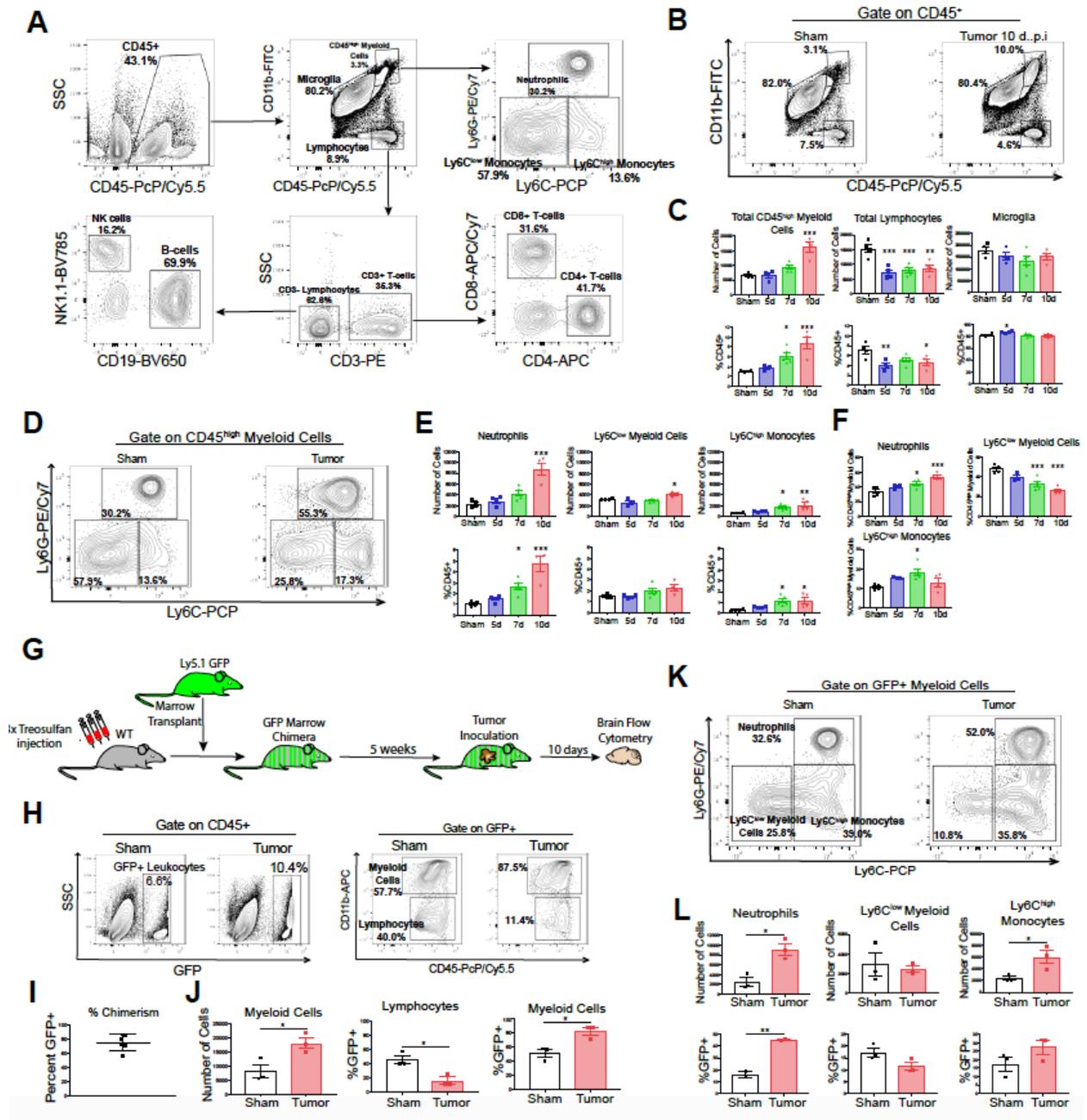

Figure 1. Myeloid cells infiltrate the brain during PDAC cachexia. A) Flow cytometry plots of immune cells isolated from whole brain homogenate, showing gating strategy to identify different immune cell populations. B) Representative flow cytometry plots

displaying CD45 and CD11b fluorescent intensities of immune cells isolated from brains of tumor and sham animals, gated on live, singlet, CD45+ cells. C) Quantification of different immune cell populations in the brain at different time points throughout the course of PDAC. d = days post inoculation. Populations were identified as shown in Fig 1a. D) Representative flow cytometry plots displaying Ly6C and Ly6G fluorescent intensities of immune cells isolated from brains of tumor and sham animals, gated on CD45^{high}CD11b^{high} cells. E) Quantification of different CD45^{high} myeloid cell populations in the brain at different time points throughout the course of PDAC. F) Relative amounts of different CD45^{high} myeloid cell populations as a percentage of total CD45^{high} myeloid cells, throughout the course of PDAC. Populations identified as described for Fig 1e. For 1c, e, and f: data are presented as mean ± s.e.m., n = 4-5/group, *P < 0.05, **P < 0.01, ***P < 0.001 compared to sham group in one-way ANOVA Bonferroni *post hoc* analysis, and results are representative of three independent experiments. G) Diagram of bone marrow transplant protocol to generate GFP+ bone marrow chimeras. H) Gating strategy for CD45+GFP+ cells isolated from brains of tumor and sham GFP chimera animals. I) Percent chimerism, identified as percentage of CD45+ cells in the blood that were GFP+. J) Quantification of GFP+ myeloid cells and lymphocytes in the brains of tumor and sham mice, 10 d.p.i. K) Representative flow cytometry plot of different GFP+ myeloid cell populations in the brains of tumor and sham GFP bone marrow chimera animals, 10 d.p.i. L) Quantification of different GFP+ myeloid cell populations in the brains of tumor and sham GFP bone marrow chimera animals, 10 d.p.i., as identified in Fig 1k. For 1j and l: data are presented as mean±s.e.m., n = 3/group, *P < 0.05, **P < 0.01 in student's t-test.

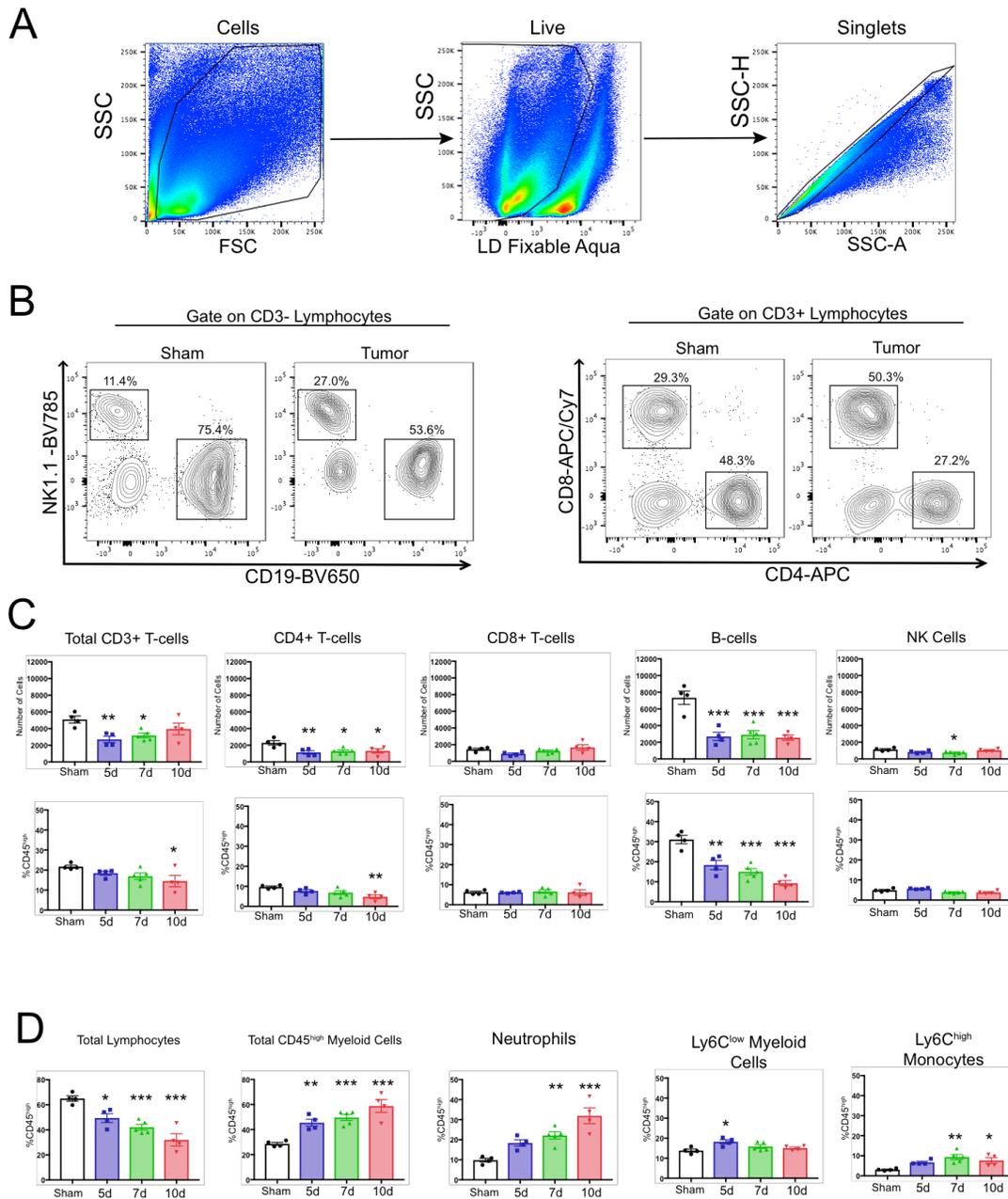**Figure S1. Decreased lymphocytes in the brain during PDAC cachexia.** A) Gating strategy to identify live single cells from whole brain homogenate. B) Representative plots of different lymphocyte populations from brain homogenate from sham and tumor (10 d.p.i.) animals. For CD3- cells, NK cells = NK1.1+CD19-, B-cells = CD19+NK1.1-. For CD3+ cells, CD4+ and CD8+ T-cells were identified. C) Quantification of different

lymphocyte populations throughout the course of cachexia. *P* < 0.05, **P* < 0.01, ***P* < 0.001 compared to sham one-way ANOVA Bonferroni *post hoc* analysis. D) Quantification of different immune cell populations in the brain throughout the course of cachexia, as a percentage of CD45^{high} cells. *P* < 0.05, **P* < 0.01, ***P* < 0.001 compared to sham. *n* = 4-5/group. Results are representative of three independent experiments.

3.2 Invading myeloid cells accumulate at CNS interfaces during PDAC cachexia

We next investigated the anatomic distribution of infiltrating myeloid cells in the CNS. We performed immunofluorescence immunohistochemistry analysis at 10 d.p.i., since all tumor-inoculated animals reliably developed cachexia at this time point, yet were not at terminal stage. In addition, our flow cytometry analysis demonstrated a robust immune cell infiltrate in the brain at 10 d.p.i. For initial analysis, we defined leukocytes as CD45+ globoid cells. Although we observed scattered CD45+ globoid cells within the parenchyma in the cortex and thalamus in tumor mice (Fig S2), we observed a robust increase in leukocytes in the meninges adjacent to the hippocampus and median eminence (ME) (Fig 2B and C). We also quantified leukocytes in the area postrema, due to its importance for appetite and weight regulation (Fig 2D). While there was an increase in overall CD45 immunoreactivity, these cells appeared ramified rather than globoid (60X inset in Fig 2D), suggesting microglia activation rather than immune cell infiltration. We did not observe any CD45+ cells in the lateral parabrachial nucleus (data not shown), which has been implicated in cancer-associated anorexia (104). This was perhaps due to its lack of proximity to a circumventricular organ or meninges.

Interestingly, we observed an increase in neutrophils (defined as myeloperoxidase [MPO] positive, CD45+ globoid cells) only in the meninges surrounding the hippocampus (Fig 2B). This layer of meninges, known as the velum interpositum (VI), is a double-layered invagination of the pia matter. This potential space is closed rostrally, communicates caudally with the quadrigeminal cistern, and is highly vascularized via a number of internal cerebral arterioles and veins. Recent studies demonstrate robust immune cell recruitment into the brain via this anatomical route after mild trauma, during CNS infection, and during CNS autoimmune disease (594-596). We verified the presence of meninges in the VI with ER-TR7 labeling, which showed infiltrating neutrophils in the VI meninges in tumor mice (Fig 2E). Neutrophils in the VI were degranulating, with MPO "blebs" present at the edge of many cells, along with extracellular MPO (Fig 2F). This phenomenon was only present in brains of tumor animals and not in brains of sham animals. We were able to confirm neutrophil identity with the plasma membrane marker Ly6G and globoid morphology (Figure S2C). Neutrophil extracellular traps (NETs) were also present in the VI, as identified by citrillunated histone H3 and MPO co-labeling (Fig 2G). We were unable to perform quantification on the number of NETs present in tumor mouse brains, due to the transient nature of these events.

In the CNS parenchyma, especially in the thalamus and cortex, we frequently observed neutrophils undergoing phagocytosis by microglia, with Iba-1+ cells extending processes around MPO+ neutrophils (Fig 2H). This supports previous studies showing that microglia protect the CNS parenchyma from neutrophil invasion during various states of inflammation (151, 597, 598).

Next, we used our GFP BMT mice to verify the peripheral origin of the CD45+ globoid cells. Sham BMT mice showed very few GFP+ cells in the brain, including the cortex and thalamus (Figure S2A), as well as the meninges (data not shown). In contrast, there was a large increase in GFP+ cells in the brains of KPC mice at 10 d.p.i. We observed a pattern of infiltrating GFP+ cells that was identical to CD45+ globoid cells in our previous experiments, with scattered GFP+ cells in the cortex and thalamus (Figure S2A and B), and accumulations of GFP+ cells in the VI and ME (Fig 2I and J). In agreement with our previous data, GFP+ cells were MPO+ in the VI (Fig 2I), but not in the meninges of the ME (Fig 2J). Most of the GFP+ cells in the ME were Iba-1+, suggesting these cells were infiltrating monocytes that differentiated into meningeal macrophages. However, we did not observe any GFP+Iba-1+ cells in the CNS parenchyma.
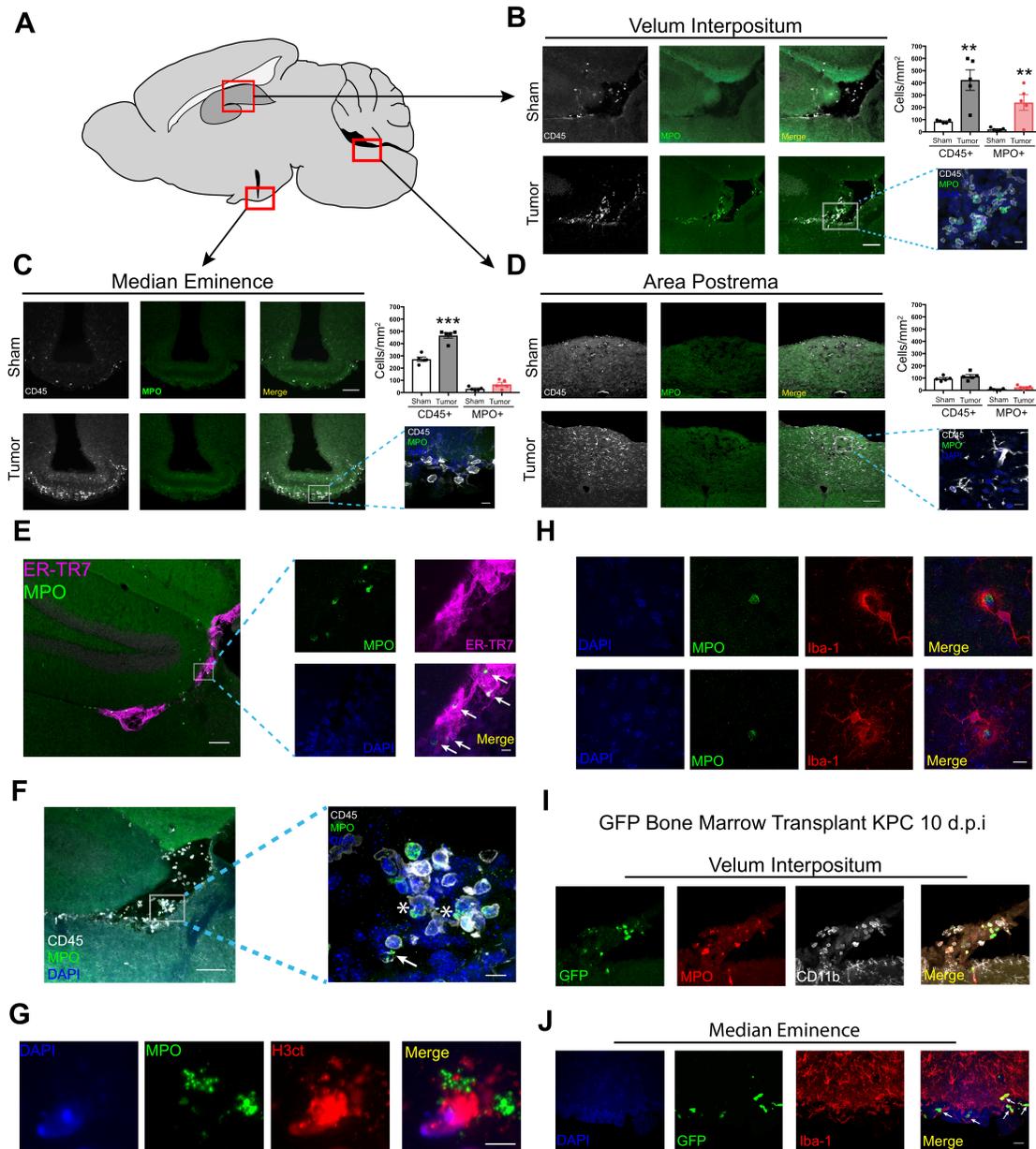
Figure 2. Infiltrating immune cells accumulate at CNS interfaces during PDAC cachexia. A) Picture of sagittal mouse brain section to illustrate different regions analyzed. B-D) 20X images of velum interpositum (B), mediobasal hypothalamus (C), and area postrema (D) of brain from sham animal and tumor animal at 10 d.p.i., with 60X inset shown on the right, along with quantification of MPO+ and total CD45+ cells. Scale bar for 20X images = 100μm. Scale bar for 60X insets = 10μm. Data are

presented as mean ± s.e.m., *n* = 5/group, *P* < 0.05, **P* < 0.01 in student's t-test. E) Representative 10X image of VI from the brain of a tumor animal 10 d.p.i., showing ER-TR7 staining to label meninges and MPO staining to label neutrophils. Scale bar = 100 μm. Inset = 60X showing neutrophils (indicated by arrows) within the meninges of the VI. Scale bar = 10 μm. F) 20X image a VI with 60X inset showing neutrophils degranulating. Asterisk = myeloperoxidase "blebs" coming off neutrophil. Arrow = extracellular myeloperoxidase. G) 60X image of neutrophil extracellular trap in the VI of a tumor animal, 10 d.p.i. Scale bar = 5 μm. H) Representative 60X images of microglia phagocytosing neutrophils in the thalamus of animals with KPC tumor, 10 d.p.i. Scale bar = 10 μm. I & J) Representative 60X images of VI and ME, respectively, from BMT GFP tumor mice, at 10 d.p.i. Scale bars = 20 μm. Arrows = GFP+Iba-1+ infiltrating macrophages.

Figure S2. Immunofluorescence analysis of infiltrating immune cells during PDAC

A) 10X confocal images of thalamus and cortex from sham and tumor mouse brains, 10 d.p.i. WT KPC = WT animals, BMT GFP = Ly5.1 eGFP marrow transplanted into WT recipient after treosulfan conditioning t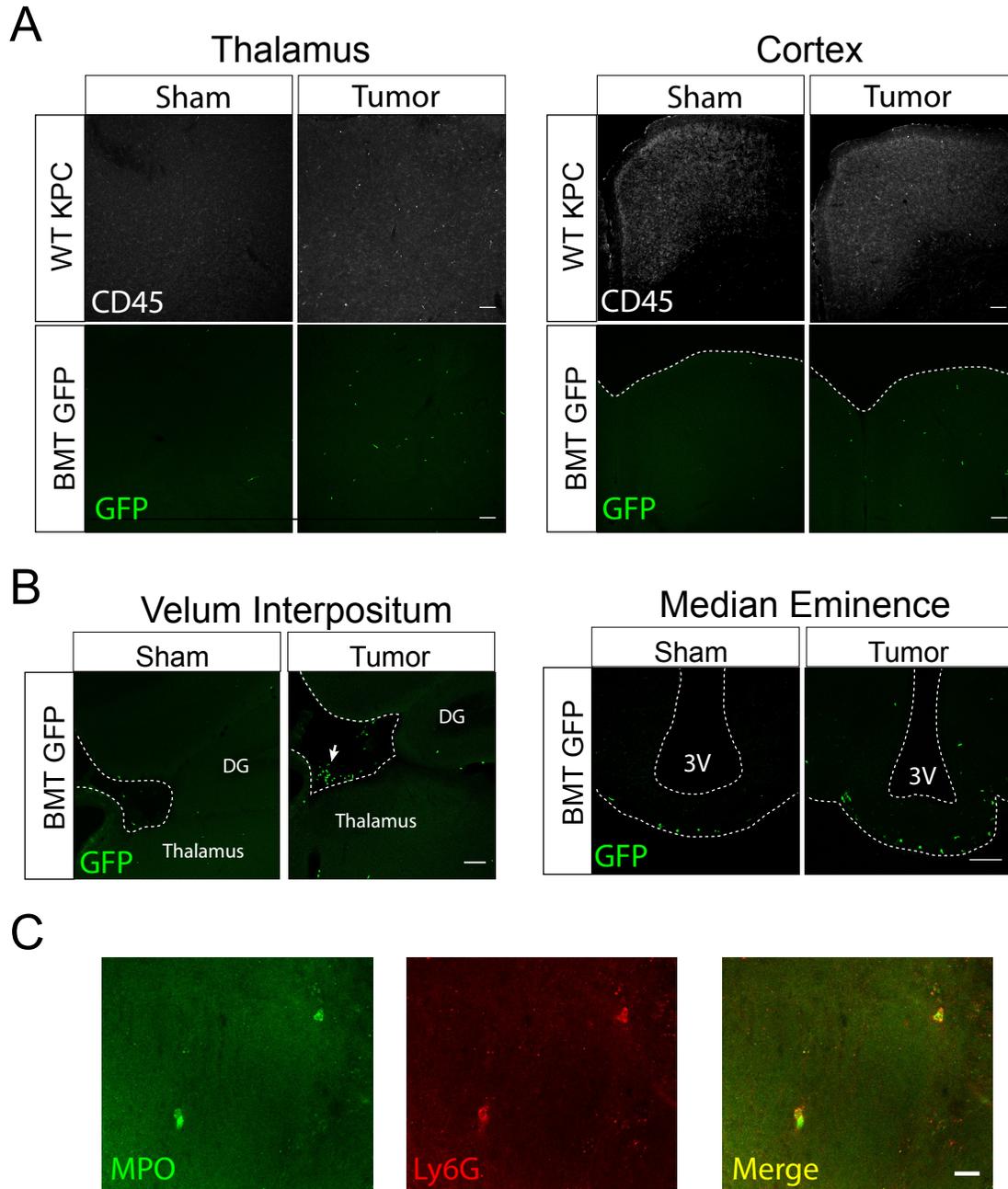o ablate marrow (see Methods). Scale bar = 100 μm. B) 10X (VI) and 20X (ME) confocal images of VI and ME from sham and tumor (10

d.p.i.) mice. In images of the VI, dashed line denotes edge of parenchyma and beginning of meninges. Arrow = cluster of infiltrating GFP+ immune cells in the VI meninges. DG = dentate gyrus. 3V = third ventricle. Scale bars = 100 μm. C) 40X confocal image of thalamus from tumor mouse, 12 d.p.i. Scale bar = 20 μm.

3.3 Behavioral and cognitive analysis during PDAC

Since we observed myeloid cell infiltration near brain regions important for behavioral and cognitive performance, we performed behavioral and cognitive tests on PDAC tumor mice to determine if these mice experienced altered anxiety levels or cognitive dysfunction. The open field test was performed on days 7 and 8 post-inoculation to assess exploratory and anxiety-like behaviors, and the object recognition test performed on days 8 and 9 post-inoculation to assess cognitive performance (Fig 3A). Overall, we observed that tumor mice were significantly less mobile than sham mice in the open field (Fig 3B and D), which is consistent with our previous home cage locomotor activity analysis showing that KPC tumor mice exhibit decreased activity throughout the course of cachexia (122). We also observed that during the open field test, tumor mice spent significantly less time in the more anxiety-provoking center of the arena compared to sham mice, indicative of anxiety-like behavior (Fig 3B and D). Lastly, despite moving significantly less than sham animals during the object recognition test, tumor animals still showed a preference for exploring the novel object compared to the familiar object (Fig 3E).
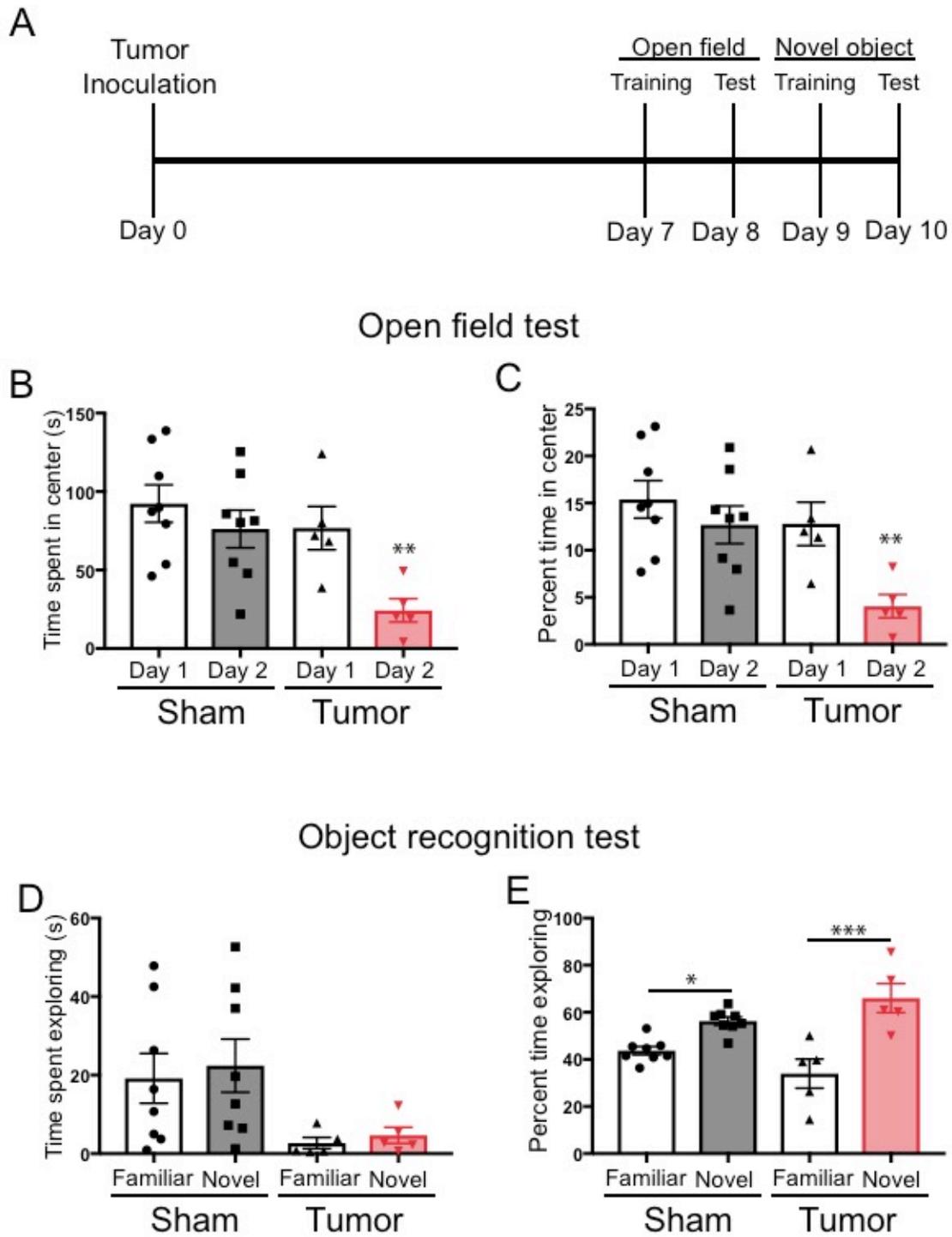
Figure 3: Cognitive performance of KPC tumor mice. A) Illustration depicting timeline of behavioral tests. B) Total time spent in center of arena during open field test

on the first and second day of testing. C) Percent of total time that was spent in the center of the arena during the first and second day of the open field test. For B and D, **P < 0.01, student's t-test comparing sham day 2 to tumor day 2. D) Total time spent investigating the familiar or the novel object. E) Percent of total time spent exploring the familiar object and the novel object. *P < 0.05, ***P < 0.001, Bonferroni post-hoc analysis in two-way ANOVA. For all panels, n = 8 sham mice and n = 5 tumor mice. Three tumor animals were excluded from all analyses due to complete lack of movement. Data are presented as mean ± s.e.m.

3.3 During PDAC cachexia the brain expresses a chemokine transcript profile that is distinct from that of the liver

Next, we investigated mechanisms of immune cell recruitment to the brain during PDAC cachexia. Using qRT-PCR, we measured relative expression levels of common chemokine transcripts in dissected hippocampi (we also included the dorsal thalamus so as to include the VI and all associated meninges) in tumor animals compared to sham animals at 10 d.p.i. We observed increased expression levels of transcripts involved with myeloid cell recruitment, including Ccl2, Cxcl1, Cxcl2 and Cxcl10 in tumor mice. Interestingly, we observed decreased levels of Cxcl5 and Cxcl9 transcript in tumor animals (Fig. 4), suggesting recruitment of specific cell types rather than a nonspecific inflammatory signal. In order to identify transcripts important specifically for immune cell recruitment to the brain, we also performed qRT-PCR on the same transcripts in the livers of tumor and sham animals. In contrast to the hippocampus, most chemokine transcripts we measured were upregulated in tumor animals, except for Cxcl5, Cxcl9, and Cxcl10. With the exception of Cxcl1 (15.4-fold increase), transcripts upregulated in

the liver showed similar levels of induction (3.6- to 4.3-fold increase) (Fig. 4). Furthermore, unlike the hippocampus, there were no chemokine transcripts that were downregulated in the livers of tumor animals. These data show that mechanisms of immune cell recruitment in the brain are different from those in the liver.
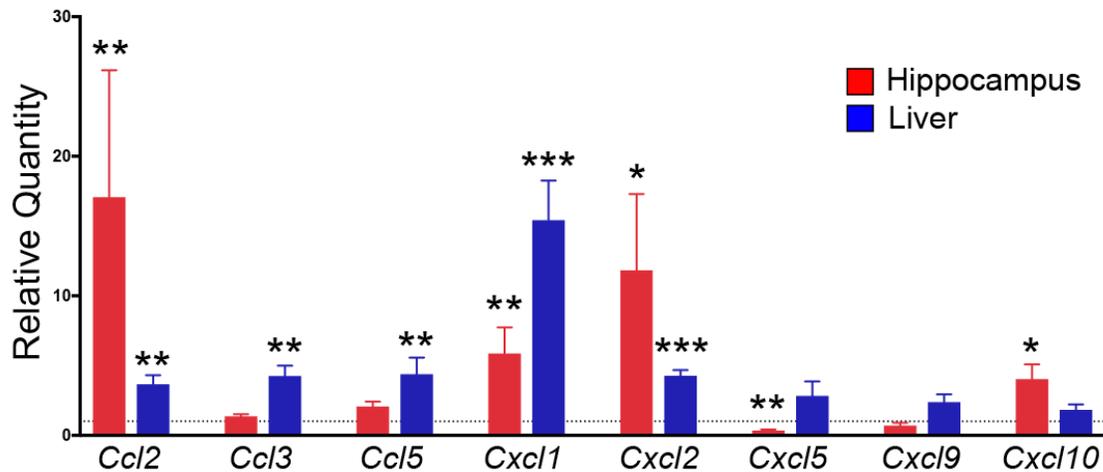
Figure 4: Distinct chemokine expression profiles in the hippocampus and liver. qRT-PCR for select chemokine transcripts from dissected hippocampi and liver from tumor and sham animals 10 d.p.i. Values are tumor relative to sham. *P < 0.05, **P < 0.01, ***P < 0.001 comparing tumor vs. sham within the same tissue type in student's t-test. n = 5/group. Results are representative of two independent experiments.

3.4 The CCR2-CCL2 axis is activated in the CNS during PDAC cachexia

We next sought to determine mechanisms of immune cell recruitment specifically to the brain during PDAC cachexia. Since *Ccl2* transcript showed the largest difference in induction between hippocampus and liver (17.1-fold vs. 3.6-fold) of all the chemokine transcripts we measured (with similar baseline levels of *Ccl2* expression, data not shown), and previous studies demonstrated the importance of CCR2/CCL2 for myeloid

cell chemotaxis to the brain (178, 599), and PDAC cachexia in humans (600), we chose to focus on the CCR2-CCL2 axis.

Using *in situ* hybridization we localized robust CCL2 mRNA expression exclusively within the VI during PDAC. There was no observable *Ccl2* mRNA in the brains of sham animals (Fig 5A). We verified these results at the protein level using CCL2^mCherry mice, which showed abundant CCL2 protein expression in the VI in tumor animals at 10 d.p.i., exclusively expressed in Iba1+CD206+ meningeal macrophages (Fig 5B). CCL2 protein was not expressed in VI meningeal macrophages in sham mice (Fig 6A). We did not observe robust CCL2 protein expression in any other locations in the brain.

Next, we assessed for the presence of CCR2 expressing cells in the brain using CCR2^RFP/WT reporter mice. We observed that, at 10 d.p.i., CCR2+ immune cells infiltrated the brains of tumor mice and accumulated in the VI (Fig 5C-E) Interestingly, a large percentage of CCR2+ cells in the VI were neutrophils (Fig 5D). CCR2+ cells were sparse or absent in other brain regions, particularly within the parenchyma, in tumor mice.

3.5 CCR2 is critical for neutrophil accumulation at CNS interfaces and cachexia during PDAC

Based on our findings that CCR2+ cells infiltrate the brain during PDAC, we hypothesized that CCR2 may play a role in cachexia. We observed that CCR2 knockout (CCR2KO) mice had decreased anorexia during PDAC cachexia compared to WT tumor mice (Fig 5F). CCR2KO tumor mice also had attenuated muscle loss compared to WT tumor mice (Fig 5G). To determine whether the decreased muscle mass loss in

CCR2KO mice was due to decreased muscle proteolysis, we assessed levels of transcripts key for muscle proteolysis in the gastrocnemius, including *Mafbx*, *Murf1*, and *Foxo1*, which we previously showed are induced by CNS inflammation (124)**.** We observed that, compared to WT tumor animals, CCR2KO tumor animals had decreased induction of *Murf1* and *Foxo1* (Fig 5H), confirming that there was decreased muscle catabolism in CCR2KO tumor mice.

We next assessed whether CCR2KO mice had attenuated immune cell infiltration in the brain using flow cytometry. We observed that at 11 d.p.i., there was a 37% decrease in total CD45^{high} myeloid cells in the brains of CCR2KO tumor mice compared to WT tumor mice (Fig 5I). This difference was primarily driven by a large decrease in brain-infiltrating neutrophils in CCR2KO tumor mice. In addition, there was also a significant decrease in brain-infiltrating Ly6C^{high} monocytes in CCR2KO tumor mice compared to WT tumor mice. Furthermore, there was a decrease in both neutrophils and Ly6C^{high} monocytes as a percentage of the CD45^{high} cells in the brain in CCR2KO tumor mice, indicating that the differences were not due to a global decrease in infiltrating immune cells (Fig 5I). This was also supported by the fact that there were no differences in microglia, Ly6C^{low} monocytes, or T-cells in the brains of CCR2KO tumor mice compared to WT tumor mice (Fig 6B and C).

Since CCR2+ immune cells, particularly neutrophils, in the brains of tumor mice localized primarily to the VI, we hypothesized that there would be a decrease in immune cells in the VI in CCR2KO tumor animals. Indeed, we observed a dramatic decrease in both total CD45+ globoid and MPO+ immune cells in the VI in CCR2KO tumor mice compared to WT tumor mice (Fig 5J and K).

Since the CCR2 deletion was not brain specific in the CCR2KO mice, we performed an extensive analysis of infiltrating immune cells in other organs. We found no differences in infiltrating neutrophils, total myeloid cells, or Ly6C^{high} monocytes in the livers of CCR2KO tumor animals compared to WT tumor animals (Fig 6D and E). Furthermore, while the tumors of CCR2KO mice were slightly smaller than those of WT mice (Fig 6F), we observed no difference in tumor immune cell composition, including neutrophils, Ly6C^{high} monocytes, Ly6C^{low} myeloid cells, or T-cells in CCR2KO mice (Fig 6G and H). When we assessed neutrophils in CCR2KO tumor mice in different organs as a percentage of those in WT tumor mice, we found the largest decrease to be in the brain (Fig 6K). Furthermore, we observed that there was a slight increase in circulating neutrophils in CCR2KO tumor mice compared to WT tumor mice (Fig 6I and J), suggesting that the decrease in brain-infiltrating neutrophils was due to a homing defect rather than inability to mobilize from the bone marrow. Lastly, although there was a 47% decrease in brain-infiltrating Ly6C^{high} monocytes in CCR2KO tumor mice compared to WT tumor mice, we also observed a 59% decrease in circulating Ly6C^{high} monocytes, suggesting that, unlike neutrophils, the decrease in brain-infiltrating Ly6C^{high} monocytes was in fact due to a defect in marrow extravasation (Fig 6K). Therefore, our data show that CCR2 is important for neutrophil recruitment specifically to the brain, and that the decrease in brain-infiltrating neutrophils was due to a homing defect rather than inability to mobilize from the bone marrow.

Taken together, these data show that the CCR2-CCL2 axis is important for cachexia during PDAC, and that this axis is required for myeloid cell recruitment specifically to the CNS.
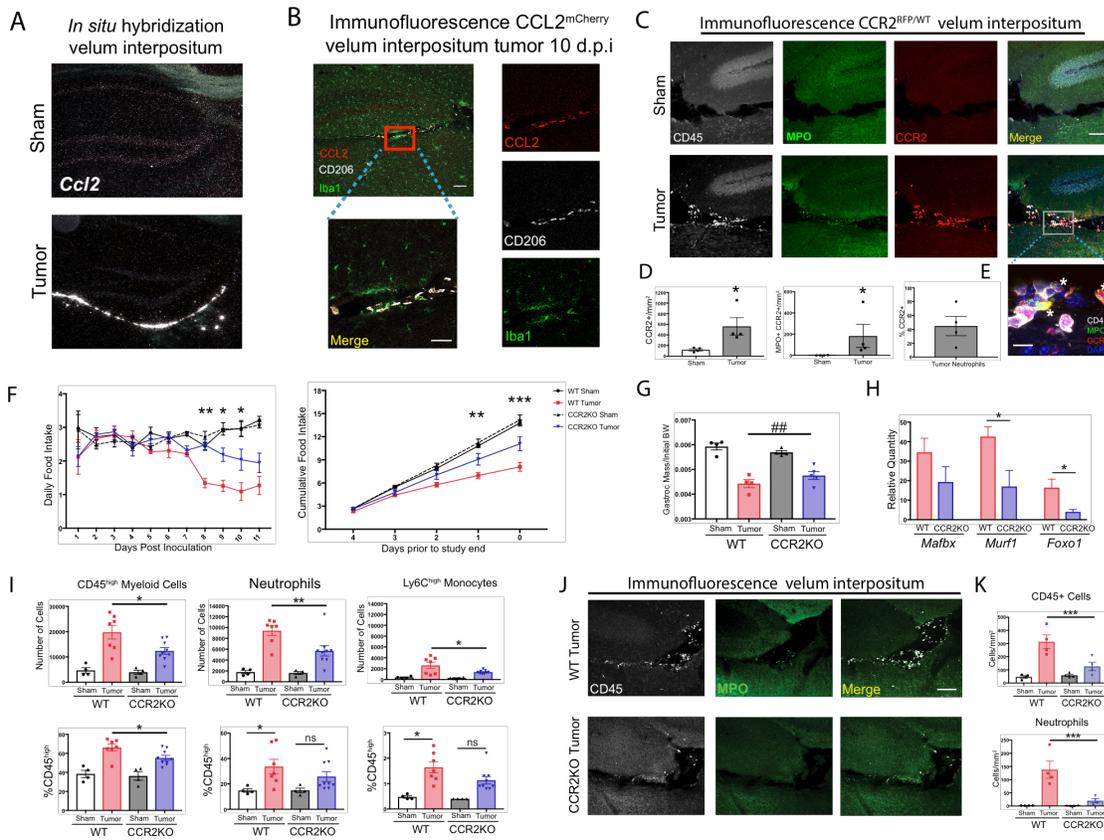
Figure 5. The CCR2-CCL2 axis in the CNS is critical for PDAC cachexia. A) Representative darkfield microscopy image of *in situ* hybridization for CCL2 in sham and tumor (10 d.p.i) mouse brains. B) Representative 10X confocal microscopy image of brain from CCL2^mCherry tumor mouse brain, 10 d.p.i. Scale bar = 100 μm. Inset of VI shows CCL2 protein expression is confined to meningeal macrophages, identified by CD206 labeling. Scale bar = 20 μm**.** C) Representative 20X confocal microscopy image of brain from CCR2^RFP/WT tumor (10 d.p.i.) and sham mouse brain. Scale bar = 100 μm. D) Quantification of different RFP+ cell populations in the VI of CCR2^RFP/WT tumor (10 d.p.i.) and sham animals. *n* = 4/group. *P < 0.05, Mann-Whitney U-test comparing sham to tumor. E) 60X inset of C identifying CCR2+ neutrophils in the VI of a tumor animal,

indicated by asterisks. Scale bar = 10 µm. F) Daily food intake (left) and final 5 days of the study (right, starting when animals develop cachexia) in WT and CCR2KO tumor and sham mice. *P < 0.05, **P < 0.01, ***P < 0.001 comparing WT tumor vs. CCR2KO tumor in Bonferroni *post hoc* analysis in two-way ANOVA. n = 4/5 per group. Results are representative of three independent experiments. G) Left = mass of dissected gastrocnemius, normalized to initial body weight, at 11 d.p.i. ##P < 0.01 for interaction effect between genotype and tumor status in two-Way ANOVA analysis. H) qRT-PCR analysis of *Mafbx*, *Murf1*, and *Foxo1* from RNA extracted from gastrocnemii dissected at 11 d.p.i. Values normalized to those from WT sham. *P < 0.05, WT tumor vs. CCR2KO tumor dCt values. n = 3-5/group. I) Flow cytometry analysis of immune cells isolated from whole brain homogenate. *P < 0.05, **P < 0.01, WT tumor vs. CCR2KO tumor, or tumor vs. sham in the same genotype in Bonferroni *post hoc* analysis in two-way ANOVA. ns = not significant. n = 4-9/group. Data consist of two independent experiments pooled (n = at least 2/group in each experiment). J) Representative 20X confocal microscopy images of the VI from WT tumor and CCR2KO tumor brain, 10 d.p.i. Scale bar = 100 µm. K) Quantification of total CD45+ globoid cells and MPO+ neutrophils in the VI of WT and CCR2KO tumor and sham animals, 10 d.p.i. ***P < 0.001, WT tumor vs. CCR2KO tumor in Bonferroni *post hoc* analysis in two-way ANOVA. n =4/group. For all figures, data are presented as mean ± s.e.m.

A Immunofluoresence CCL2^mCherry velum interpositum sham

Brain

B Gate on CD45^high myeloid cells

C Ly6C^low Myeloid Cells / T-cells

Liver

D Gate on myeloid cells

E Ly6C^low Myeloid Cells / Ly6C^mid Myeloid Cells / Ly6C^high Monocytes / Neutrophils / T-cells

Tumor

F

G Gate on myeloid cells

H

Blood

I Gate on myeloid cells

J Ly6C^low Myeloid Cells / Ly6C^high Monocytes / Neutrophils / T-cells

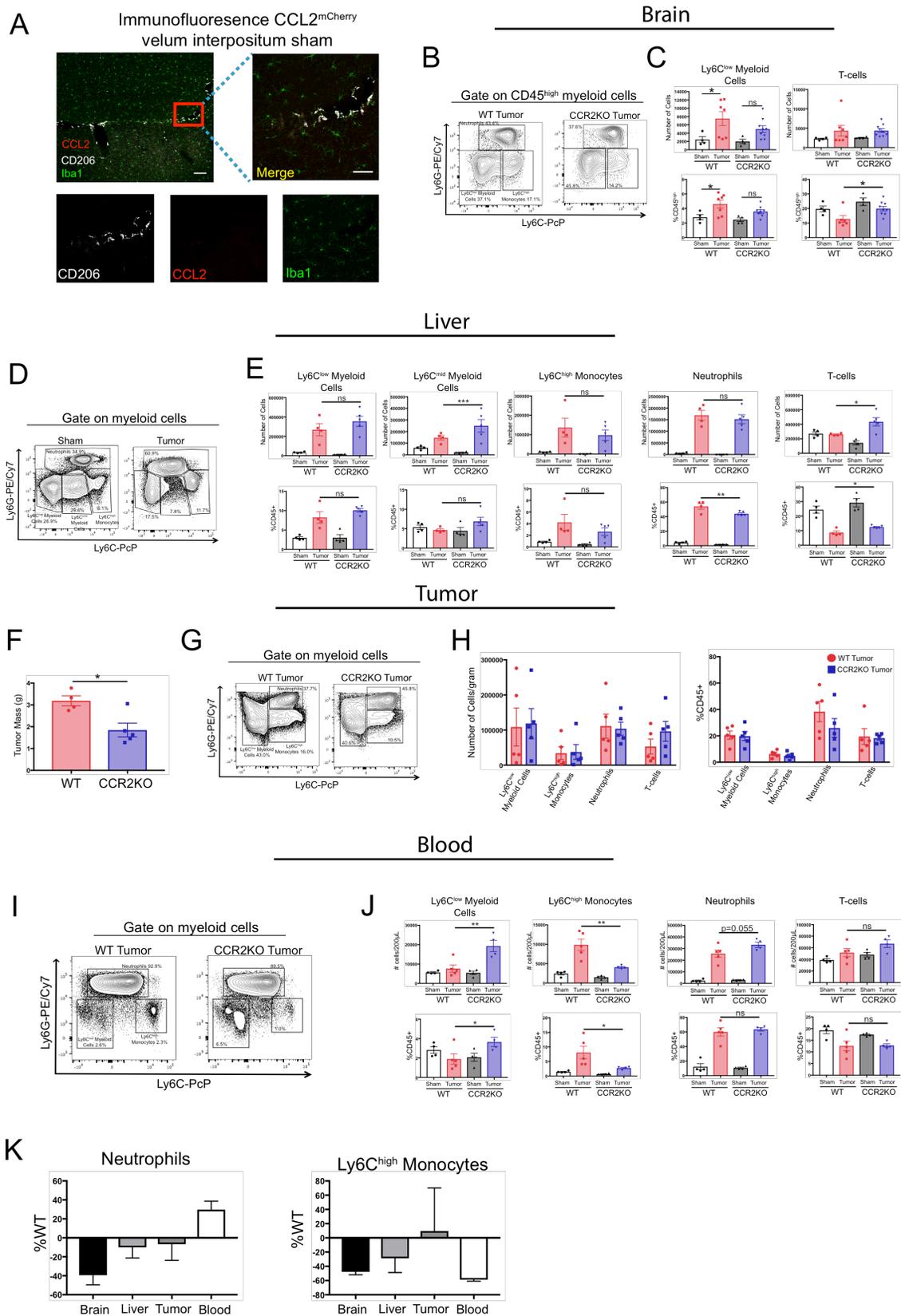**K** Neutrophils / Ly6C^high Monocytes

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Figure 6. The CCR2-CCL2 axis is of selective importance for the brain in PDAC cachexia. A) Representative 10X confocal microscopy image of brain from CCL2^{mCherry} sham mouse brain. Scale bar = 100 µm. Inset scale bar = 20 µm. B) Representative plot of different CD45^{high} myeloid cell populations from WT and CCR2KO tumor animal brains, 11 d.p.i. Cells are gated on live, singlet, CD45+, CD45^{high}CD11b+ cells. C) Quantification of Ly6C^{low} myeloid cells and T-cells from WT and CCR2KO tumor and sham animal brains. *$P < 0.05$, WT tumor vs. CCR2KO tumor, or tumor vs. sham in the same genotype in Bonferroni *post hoc* analysis in two-way ANOVA. ns = not significant. n = 4-9/group. Results consist of two independent experiments pooled (n = at least 2 per group in each experiment). D) Representative flow cytometry plot of different myeloid cell populations from WT sham and tumor livers, 11 d.p.i, in order to illustrate different myeloid cell populations identified based on Ly6C and Ly6G expression. Cells are gated on live, singlet CD45+CD11b+ cells. E) Quantification of flow cytometry analysis of different immune cell populations in the liver from WT and CCR2KO sham and tumor animals, 11 d.p.i. *$P < 0.05$, **$P < 0.01$, WT tumor vs. CCR2KO tumor, or tumor vs. sham in the same genotype in Bonferroni *post hoc* analysis in two-way ANOVA. ns = not significant. n = 4-9/group. F) Tumor mass from WT and CCR2KO animals, 11 d.p.i. Data are representative of three independent experiments. Data are presented as mean ± s.e.m. G) Representative flow cytometry plot of different myeloid cell populations from WT and CCR2KO tumors, 10 d.p.i. Cells are gated on live, singlet CD45+CD11b+ cells. H) Quantification of flow cytometry analysis of different immune cell populations isolated from tumor from WT and CCR2KO tumor animals, 10 d.p.i. Data consist of two independent experiments pooled (n = at least 2 per group per

experiment). Data are presented as mean ± s.e.m. I) Representative plot of different myeloid cell populations from WT and CCR2KO tumor animal blood, 10 d.p.i. Cells are gated on live, singlet CD45+CD11b+ cells. J) Quantification of flow cytometry analysis of different immune cell populations in the blood from WT and CCR2KO sham and tumor animals, 10 d.p.i. *$P < 0.05$, **$P < 0.01$, WT tumor vs. CCR2KO tumor, or tumor vs. sham in the same genotype in Bonferroni *post hoc* analysis in two-way ANOVA. ns = not significant. *n* =4-5/group. Data are representative of two independent experiments. K) Analysis of neutrophils and Ly6C^{high} monocytes in brain, liver, tumor, and blood in CCR2KO tumor mice, normalized to number in WT tumor mice. *n* = 5-9/group.

3.6 Blockade of P2RX7 in the CNS prevents immune cell infiltration into the brain and attenuates cachexia during PDAC

To evaluate the effects of CNS inflammatory responses independent of effects on tumor biology, we directly assessed whether immune cell infiltration into the brain mediates cachexia by treating mice with intracerebroventricular (ICV) oxidized ATP (oATP). This potently blocks purinergic receptor P2RX7 signaling on brain resident macrophages. Signaling through this receptor is key for neutrophil recruitment to the brain during neuroinflammation (601). Animals were surgically implanted with indwelling lateral ventricle cannulas, then inoculated with KPC cells one week later. Mice received daily ICV injections of either 500 ng oATP or vehicle (aCSF), starting 3 d.p.i. (Fig 7A). We observed that oATP treatment attenuated anorexia in tumor mice (Fig 7B). We also observed a trend toward increased gastrocnemius mass (*P* = 0.09) in oATP-treated tumor mice compared to aCSF-treated tumor bearing mice (Fig 7C), which corresponded to a trend toward decreased induction of genes associated with

proteolysis in gastrocnemius muscle (Fig 7D), demonstrating that muscle catabolism was moderately attenuated by oATP administration directly into the brain. Tumor size in oATP-treated tumor mice was identical to that of aCSF-treated tumor mice (Fig S3A). Furthermore, oATP treatment completely prevented both neutrophils and total CD45^{high} myeloid cells from infiltrating the brain (Fig 7E and F). Ly6C^{low} myeloid cells and T-cells were not affected (Fig S3B). Furthermore, ICV oATP treatment did not affect any circulating immune cell population (Fig S3C). When we investigated infiltrating immune cells in the VI, both CD45+ globoid cells and CD45+MPO+ neutrophils were completely absent in oATP-treated tumor animals, compared to large infiltrates in aCSF-treated tumor animals (Fig 7G). While we did observe sparse CD45+ cells in the VI in oATP-treated tumor animals, they were not globoid and resembled meningeal macrophages.

Since ICV oATP antagonizes P2RX7 on brain macrophages, we investigated its effect on microglia. To quantify activation state we assessed microglia morphology in the hippocampus. We did not observe any differences in microglia size, Iba-1 staining area, and Iba-1 intensity per cell when comparing aCSF- or oATP-treated tumor animals to oATP-treated sham animals or each other (Fig 7I and J). These results show that microglia activation state in the hippocampus is not affected by the presence of a pancreatic tumor or oATP administration to the brain.
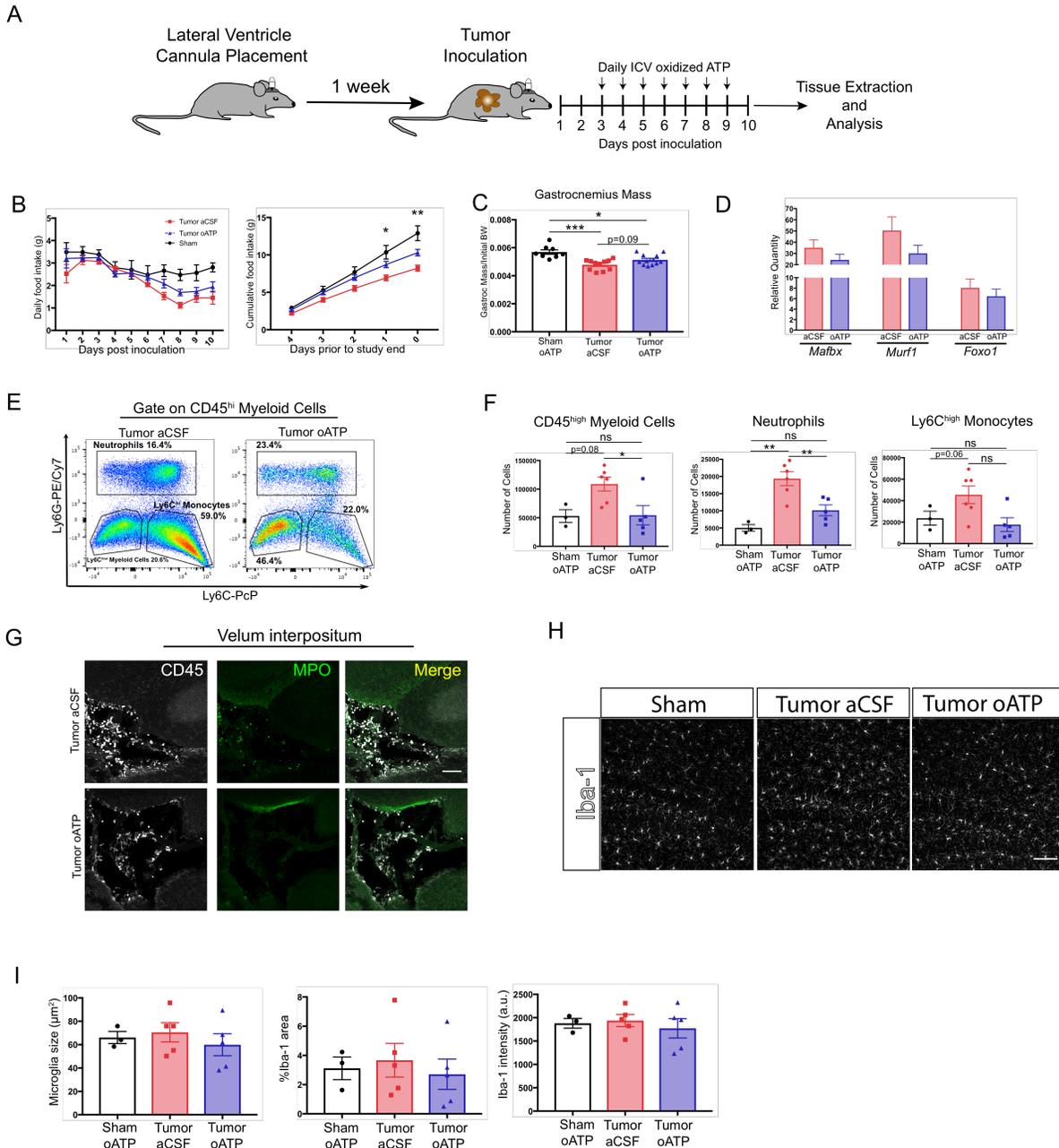
Figure 7. Intracerebroventricular administration of oxidized ATP prevents immune cell recruitment to the brain and attenuates cachexia during PDAC. A) Diagram depicting workflow for lateral ventricle cannulation and ICV oATP treatment during PDAC. ICV = intracerebroventricular. B) Daily food intake (left) and cumulative food intake for the final 5 days of the study (right, starting when animals develop cachexia)

P < 0.05, **P* < 0.01, comparing aCSF tumor vs. oATP tumor in Bonferroni *post hoc* analysis in two-way ANOVA. *n* = 8-12/group. Results consist of two independent experiments pooled (*n* = 4-7/group in each experiment). C) Left = mass of dissected gastrocnemius, normalized to initial body weight, at 10 d.p.i. D) qRT-PCR analysis of *Mafbx*, *Murf1*, and *Foxo1* from RNA extracted from gastrocnemii dissected at 8-10 d.p.i. Values normalized to those from sham oATP. *n* = 4-7/group. E) Representative flow cytometry plot of CD45^{high} myeloid cells from brain of tumor aCSF and tumor oATP mice. F) Quantification of immune cells isolated from whole brain homogenate. *P* < 0.05, **P* < 0.01, in Bonferroni *post hoc* analysis in two-way ANOVA. ns = not significant. *n* = 4-7/group. G) Representative 20X confocal microscopy images of the VI from aCSF-treated and oATP-treated tumor animals. Scale bar = 100 µm. H) Representative 20X images of Iba-1 immunofluorescence in the dentate gyrus, 10 d.p.i. Scale bar = 100 µm. I) Quantification of microglia morphology in the dentate gyrus 10 d.p.i., showing mean microglia size (left), percent area covered by Iba-1 immunofluorescence (middle), and mean Iba-1 fluorescent intensity per microglia (right). a.u. = arbitrary units. *n* = 3-5/group.
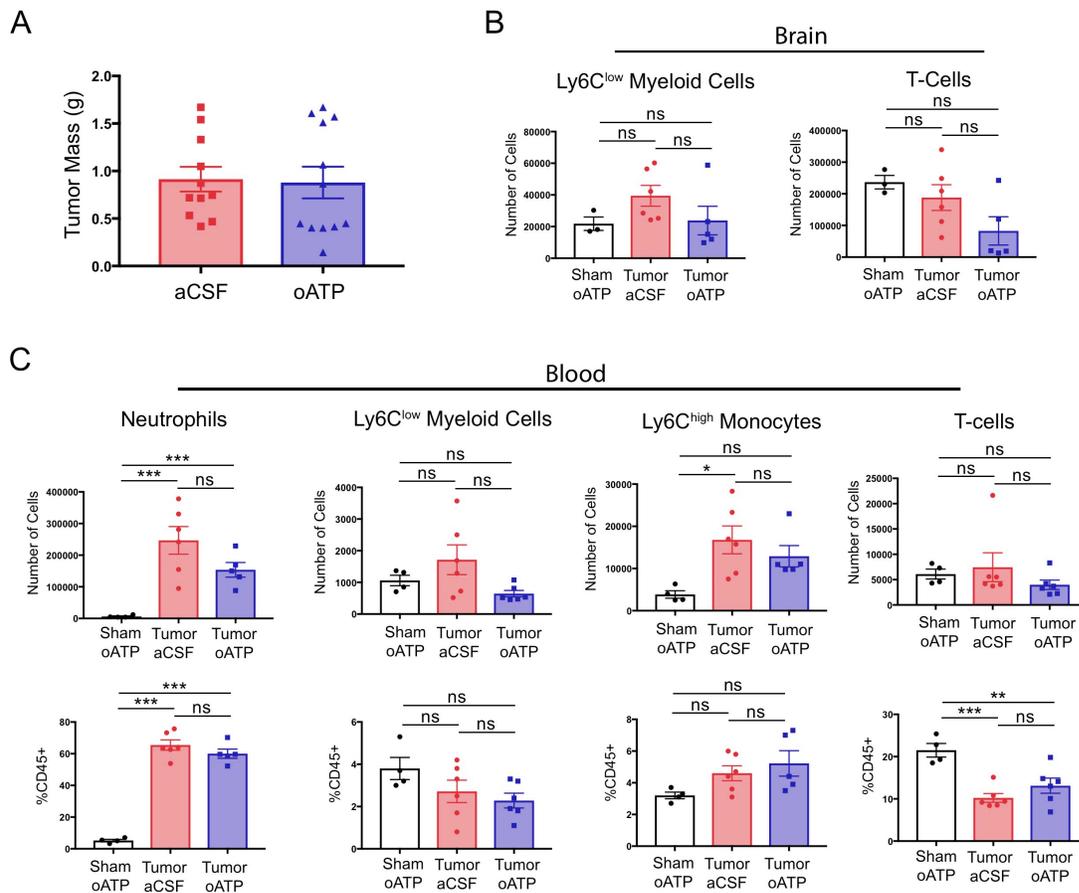
Figure S3. Intracerebroventricular antagonism of P2RX7 does not affect systemic inflammation or tumor size during PDAC. A) Tumor mass from aCSF- and oATP-treated tumor-bearing mice, 8-10 d.p.i. *n* = 11-12/group. Results consist of two independent experiments pooled (*n* = 5-7/group in each experiment). B) Quantification of immune cells isolated from whole brain homogenate. ns = not significant in Bonferroni *post hoc* analysis in two-way ANOVA. *n* = 4-7/group. C) Quantification of immune cells isolated from blood, per 200 μL of blood. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in Bonferroni *post hoc* analysis in two-way ANOVA. ns = not significant. *n* = 4-6/group.

3.8 Brain-infiltrating neutrophils express a transcriptional profile that is distinct from circulating, liver-, and tumor-infiltrating neutrophils during PDAC.

Based on our data showing that CCR2 is a brain-specific chemotactic receptor for neutrophils during PDAC, we hypothesized that brain-infiltrating neutrophils are unique compared to neutrophils that infiltrate other organs. In addition, it was previously shown that the CNS microenvironment can shape the function of infiltrating myeloid cells in a way that is distinct from other tissues (602). Therefore, in order to characterize the phenotype of brain-infiltrating neutrophils during PDAC, we performed RNA sequencing (RNAseq) on FACS-sorted neutrophils from blood, liver, tumor, and brain during PDAC at 10 d.p.i, as well as circulating neutrophils from sham animals (Fig 8A). Principal component analysis of individual samples based on the top 500 most varying transcripts revealed that brain-infiltrating neutrophils clustered tightly together, but were distinct from those in liver, tumor, and blood (Fig 8B). We identified putative "brain-specific" transcripts by comparing the transcriptome of brain-infiltrating neutrophils to that of liver- and tumor-infiltrating neutrophils, as well as circulating neutrophils (all from tumor animals). In order to control for the nonspecific effects of malignancy on circulating neutrophils, we any excluded transcripts that were upregulated in circulating neutrophils from tumor animals compared to circulating neutrophils from sham animals. Using this approach, we identified 104 upregulated and 126 downregulated "brain-specific" transcripts (Fig 8C and D). Functional enrichment analysis (based on Gene Ontology curation) of brain-specific transcripts identified enrichment for the term "extracellular space" (GO:0005615) in upregulated genes and enrichment for the terms "external side of plasma membrane" (GO:0009897), "immune response" (GO:0006955), and "response to interferon-gamma" (GO: 00034341) in downregulated genes. Several

brain-specific upregulated transcripts encoded neutrophil granule components and enzymes (Fig 8E and Fig S4), such as neutrophil granule protein (*Ngp*), the metalloproteinase ADAMTS5 (*Adamts5*) neutrophil elastase (*Elane*), lactoferrin (*Ltf*), cathelicidin antimicrobial peptide (*Camp*), and transthyretin (*Ttr*), as well as proteins important for granule secretion and NET formation such as dynamin 3 (*Dnm3*), synaptotagmin 15 (*Syt15*), Serpinb1a (*Serpinb1a*), Serpin Family E Member 2 (*Serpine2*), C/EBPε (*Cebpe*) (603), and Myosin VIIA And Rab Interacting Protein (*Myrip*) (604). We also observed an increase in genes for solute carriers (*Slc* gene family) and components of the Na/K ATPase, suggesting brain-infiltrating neutrophils are highly metabolically active. Many brain-specific downregulated transcripts encoded proteins important for immune function and responsiveness to T-cell-derived cytokines, such as MHC II (*H2-Ab1*), CXCL7 (*Ppbp*), PDLIM1 (*Pdlim1*, a negative regulator of NFκβ signaling), and the interferon-inducible genes *Ifi203*, *Ifi205*, and *Ifi209* (Fig 8E and Fig S4). Taken together, these data indicate that the CNS microenvironment uniquely influences the neutrophil transcriptome during PDAC.
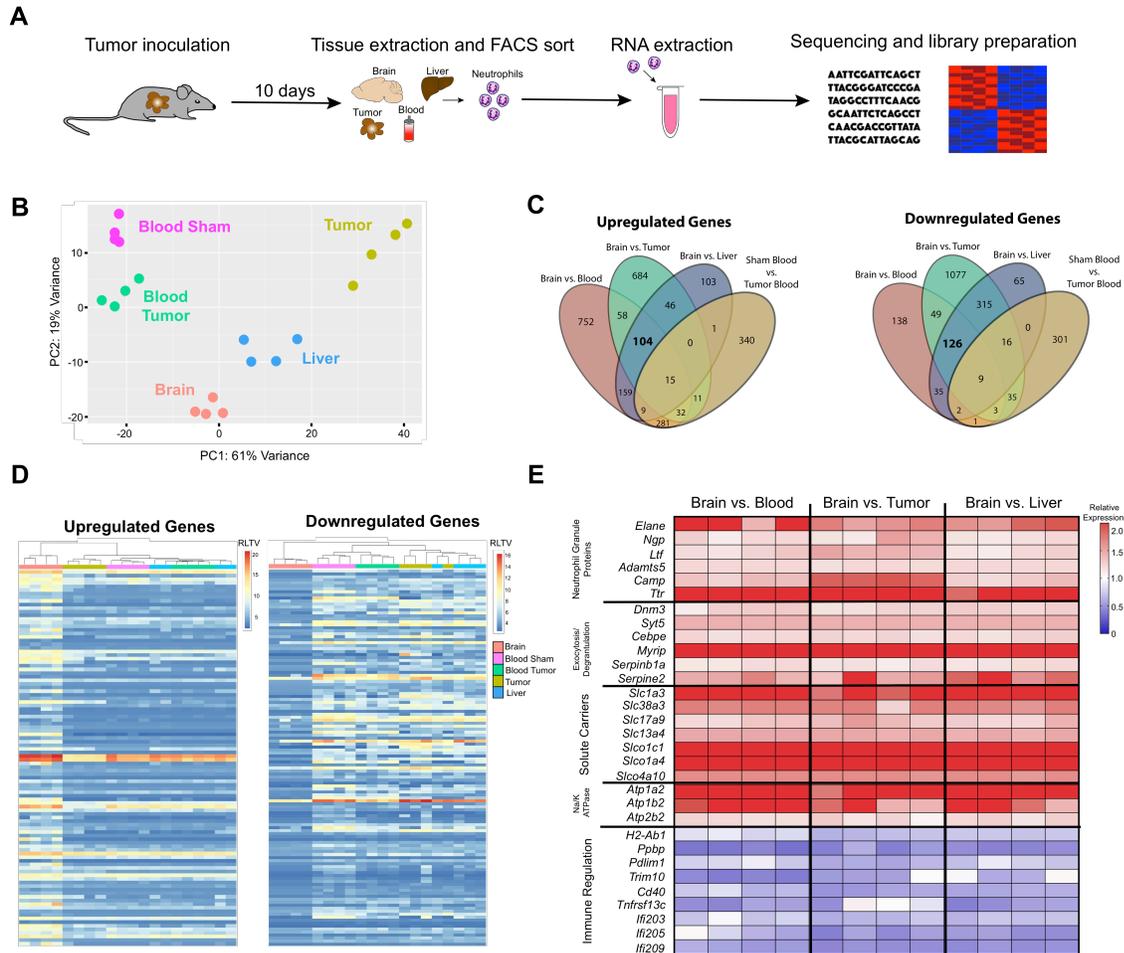
Figure 8. Brain-infiltrating neutrophils express a unique transcriptome during PDAC. A) Workflow for neutrophil isolation, RNA extraction, and RNAseq analysis. B) Principle component analysis of 500 most varying genes in neutrophils isolated from blood, tumor, liver, and brain from mice with PDAC at 10 d.p.i., as well as blood from sham mice. C) Venn diagram of different comparisons of transcripts expressed in neutrophils from different organs. D) Heatmaps of "brain-specific" transcripts, showing transcript expression. RLTV = regularized logarithm transformed value. E) Heatmap of select brain-specific transcripts showing relative expression, comparing average of brain neutrophils to neutrophils in different organs.
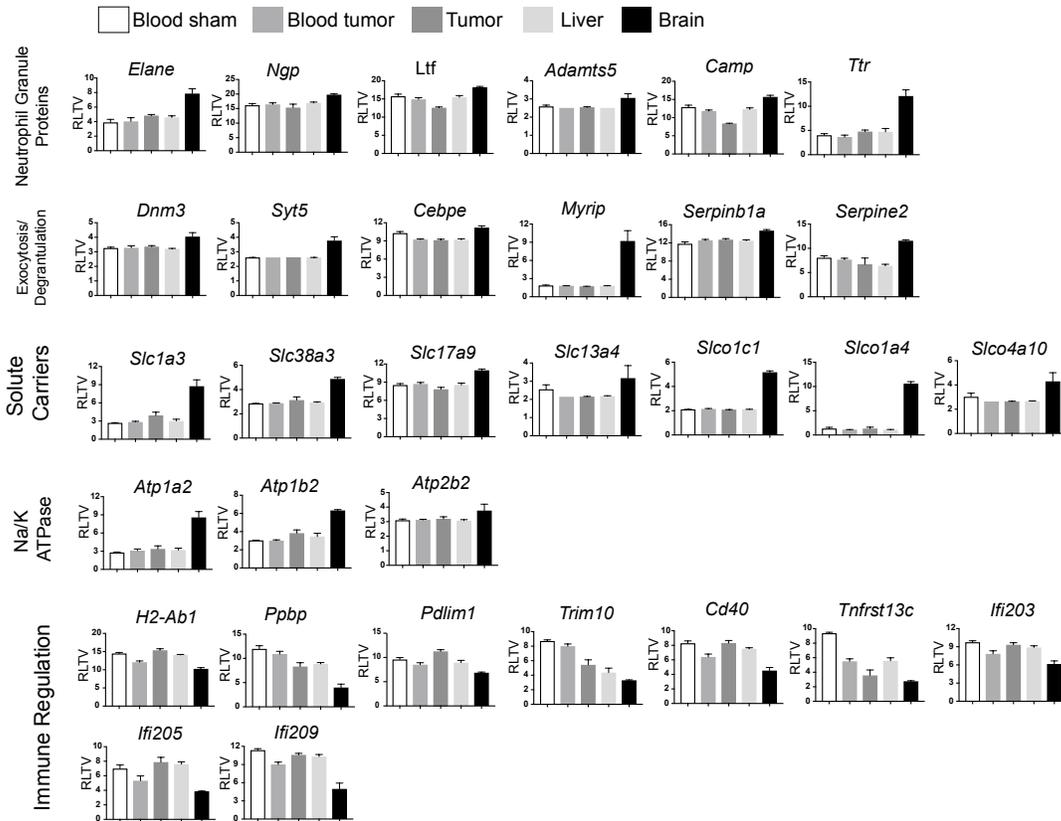
Figure S4. Expression of neutrophil brain-specific transcripts. Normalized expression values of transcripts depicted in heatmap in Figure 7e. RLTV = regularized logarithm transformed value.

4. **Discussion**

Several lines of investigation show that production of inflammatory mediators in the brain correlates strongly with cachexia during cancer (122, 123), yet the role of neuroinflammation in this syndrome is still not well understood. Our data show that in a mouse model of PDAC, myeloid cells, consisting predominately of neutrophils, infiltrate the brain, in a CCR2-dependent manner, where they drive cachexia. We observed that infiltrating immune cells accumulated specifically in a unique layer of meninges called the velum interpositum (VI), which is adjacent to the hippocampus and the habenula, the latter of which is important for appetite regulation and is associating with cachexia in humans (605). We observed robust CCL2 mRNA and protein expression, along with CCR2+ neutrophils, exclusively in this region. The VI is implicated as a key structure for initial immune infiltration during states of neuroinflammation such as EAE (596) and traumatic brain injury(595). Indeed, the VI contains the pial microvessels that are a key aspect of the "gateway reflex", a neuro-immune pathway that involves interactions between leukocytes and neurons involved in stress response (606) and is implicated in gastrointestinal dysfunction during EAE (208). While we observed myeloid cell infiltration throughout the VI, we also observed accumulation of neutrophils and other leukocytes around the same pial vessels involved in the gateway reflex. The role of the gateway reflex in feeding behavior has not been investigated. It is possible that, in our model of PDAC-associated cachexia, brain infiltrating neutrophils were involved in generating anorexia and muscle catabolism via a neuro-immune circuit similar to the gateway reflex, involving inflammation generated in the VI, and possibly transmitted to the habenula, or other regions involved in appetite regulation.

The role and presence of infiltrating leukocytes in the CNS during systemic inflammation remain poorly understood. While previous reports show that neutrophils infiltrate the brain after septic doses of LPS or sepsis induced by cecal ligation (232), it is still unknown if they contribute to neurologic sequelae (anorexia, fatigue, cognition and memory deficits, etc.) during and after sepsis. A series of studies utilizing a mouse model of inflammatory liver disease showed that "sickness behaviors" could be attenuated if myeloid cell recruitment to the brain was abrogated via any one of several different interventions, including: 1) administration of a P-selectin inhibitor (179), 2) deleting CCR2 (178), and 3) inhibiting microglia activation with minocycline (237). However, unlike our study, these studies did not address many of the signs and symptoms of cachexia, including anorexia and muscle catabolism, instead using social interaction as their sole measure of sickness behaviors. They also did not address whether their interventions affected monocyte infiltration in other tissues. In addition to systemic inflammatory diseases, Zenaro et al. showed that transient neutrophil depletion led to substantially improved amyloid beta burden, decreased neuroinflammation, and lessened cognitive decline in a mouse model of Alzheimer's disease (216). Therefore, our results, along with previous studies, implicate brain-infiltrating myeloid cells as key players in driving CNS-mediated signs and symptoms during inflammatory disease.

We observed a decrease in total number of lymphocytes in the brain starting at 5 d.p.i., which persisted throughout the course of cachexia. This was driven by a decrease in B-cells and CD4+ T-cells. Since the vast majority of lymphocytes in the non-inflamed murine brain are intravascular, even after thorough perfusion of the

vasculature (139), we chose not to pursue lymphocytes in our subsequent analysis. However, these interesting results warrant investigation of the role of intravascular lymphocytes in the brain during conditions of inflammation. While several studies showed that intravascular neutrophils can induce pathology in the brain (574, 575), the function of intravascular T-cells, B-cells, and NK cells, which are presumably adherent to the endothelium, has yet to be investigated.

We performed the open field test and object recognition test to assess behavioral alterations and cognitive injury in KPC-derived tumor mice. We observed that tumor-bearing mice spent significantly less time in the center of the arena during the open field test, indicative of anxiety-like behavior. Our results are consistent with previous studies on animals inoculated with Lewis lung carcinoma cells, showing that tumor animals display anxiety-like behaviors (104, 607). However, it is important to note that tumor animals moved significantly less than sham animals. Therefore, the severe decrease in locomotor activity animals experienced may affect the ability to detect alterations in anxiety-like behavior and complicates extensive behavioral analysis in this model. The preferential exploring of the novel object in the object recognition test indicates that in contrast to activity, cognition was not impaired in this model.

We showed that CCR2KO mice exhibited significantly attenuated myeloid cell infiltration into the brain and cachexia during PDAC. As discussed above, these results are in agreement with previous studies investigating sickness behaviors during inflammatory liver disease, which showed that CCR2KO mice exhibited attenuated monocyte infiltration into the brain, along with decreased sickness behaviors (178). Furthermore, it was recently reported that mice lacking CCR2 had decreased myeloid

cell infiltration into the brain and attenuated cognitive impairment during a model of sepsis induced by *Streptococcus pneumoniae* injection into the lungs (608). In an attempt to identify inflammatory biomarkers for PDAC-associated cachexia, Talbert et al. identified CCL2 as the only cytokine or chemokine (out of a panel of 25) that was increased in the serum of cachectic PDAC patients but not increased in the serum of non-cachectic patients (600). While CCR2 is usually not considered a key receptor for neutrophil recruitment, previous studies show it is important for neutrophil chemotaxis during sepsis (609, 610). Interestingly, while we observed a robust decrease in brain-infiltrating neutrophils, we did not observe a decrease in liver- or tumor-infiltrating neutrophils in CCR2KO tumor mice, indicating that CCR2 is important for neutrophil recruitment specifically to the brain. In addition, there was actually a small increase in circulating neutrophils in CCR2KO tumor mice, ruling out the possibility that neutrophils were unable to extravasate out of the marrow. Therefore, our findings demonstrate a novel role for CCR2 in neutrophil recruitment to the brain during systemic inflammation and show that the CCR2-CCL2 axis is critical for cachexia during systemic inflammation.

We administered oxidized ATP, a purinergic receptor antagonist, directly into the brain and observed attenuated cachexia and complete abrogation of circulating myeloid cell recruitment to the brain in tumor animals. These results provide key mechanistic insights to show that brain inflammation is key for pancreatic cancer cachexia. While there was no change in microglia morphology after oATP administration, consistent with previous studies (601, 611), we cannot rule out the possibility that the differences in cachexia we observed were due to changes in microglia phenotype. The presence of an indwelling lateral ventricle cannula may have also induced microglia activation and

influenced morphology quantification. However, we did take care to acquire images from the contralateral hemisphere.   Furthermore, we observed an increased Ly6C^{high} monocyte infiltrate in our aCSF-treated tumor animals compared to non-cannulated tumor animals, suggesting the indwelling lateral ventricle cannula did affect the inflammatory response in the brain to at least a small degree. Nevertheless, oATP completely prevented myeloid cells from infiltrating the brain during PDAC, strongly implicating these cells as mediators of cachexia.

A few limitations should be considered when interpreting results of this study. First, our data was produced in a single model of pancreatic cancer cachexia. While our model is extensively characterized and reliably recapitulates all of the cardinal features of cachexia seen in humans, other disease models with associated cachexia (heart failure, rheumatoid arthritis, etc.) should also be considered. Second, it is possible, even likely, that circulating immune cells infiltrate and influence function in other organs involved in cachexia (skeletal muscle, adipose tissue, etc.). However, the purpose of this study was to investigate and characterize interactions between circulating immune cells and the brain during PDAC. Therefore, we chose to focus specifically on the brain so as to not overcomplicate analysis. Third, despite our extensive analysis, we cannot rule out with absolute certainty that the differences in cachexia we saw in CCR2KO mice were not due to differences in tumor response. However, both the CCR2/CCL2 axis and neutrophils are reported to be "pro-tumor" (612, 613) and therefore systemic treatment targeting neutrophils or the CCR2/CCL2 axis in humans may be particularly beneficial in that they decrease tumor size and abrogate cachexia. This would be advantageous to conventional anti-tumor therapies such as chemotherapy and

checkpoint inhibitors, which are both known to cause cachexia-like symptoms (275, 614).

In summary, we demonstrated that myeloid cells infiltrate the CNS throughout the course of PDAC and that preventing myeloid cells from infiltrating the brain attenuates cachexia. We showed there are distinct mechanisms for immune cell recruitment to the brain during systemic inflammation, and demonstrate a novel role for CCR2 in neutrophil recruitment to the brain, providing key insights into mechanisms of neuroinflammation and cachexia.

Chapter 5. Resident Macrophages in the Hypothalamus Respond to Tumor-Derived Factors and are Protective Against Cachexia During Pancreatic Cancer

A manuscript in preparation for submission to *Glia*

Burfeind KG, Zhu X, Norgard MA, Levasseur PR, Michaelis KA, Olson B, Marks DL. 2019. Resident macrophages in the hypothalamus respond to tumor-derived factors and are protective against cachexia during pancreatic cancer.

Abstract

Microglia in the mediobasal (MBH) hypothalamus respond to inflammatory stimuli and metabolic perturbations to modulate body composition. This concept is well studied during high fat diet induced obesity, yet has not been investigated during cachexia, a devastating metabolic syndrome characterized by anorexia, fatigue, and muscle catabolism. We showed that microglia accumulated specifically in the MBH early in pancreatic ductal adenocarcinoma (PDAC)-associated cachexia and assumed an activated morphology. Astrogliosis also occurred in the MBH and hippocampus concurrent with cachexia initiation. Microglia depletion resulted in increased anorexia, fatigue, and muscle catabolism. PDAC induced microglia to express arginase-1, both *in vitro* and *in vivo*. These results demonstrate that microglia respond to tumor-derived factors and suggest this response is protective against cachexia.

1. Introduction

Cachexia is a chronic disease-associated metabolic syndrome consisting of anorexia, decreased locomotor activity, and tissue catabolism (30). It accompanies several diseases, such as cancer, rheumatoid arthritis, and Alzheimer's disease (615), but is particularly prevalent during pancreatic ductal adenocarcinoma (PDAC) (539), a neoplasm of the exocrine pancreas with a 7% five-year survival. During PDAC, cachexia is associated with increased mortality (539), decreased quality of life (616), and decreased tolerance to chemotherapy (41). Despite this serious clinical concern, there are currently no effective treatments for PDAC-associated cachexia.

Mechanisms of cachexia remain unclear, but all of the physiological processes dysregulated in this syndrome are controlled to at least some degree by the central nervous system (CNS). Systemic inflammation, which is well documented in PDAC (617), can be sensed in areas of the CNS important for metabolic function, particularly the mediobasal hypothalamus (MBH) (253, 618). Inflammatory signaling mediators (e.g., toll-like receptor agonists, cytokines, etc.) can cause altered neuronal activity in this region, leading to signs and symptoms similar to those observed in cachexia (267). Furthermore, inflammatory cytokines are produced in the hypothalamii of mice with PDAC, and deletion of inflammatory signaling mediators results in attenuated cachexia (123, 129). However, the cellular source of inflammatory mediators in the MBH is not known and remains a topic of active investigation.

Microglia, the resident macrophages of the CNS, play a key role in regulating metabolic function via their activity within the MBH (619). Microglia in this region are able to sense various circulating metabolites and in turn influence neuronal activity

(620). The role of microglia in mediating metabolic dysfunction during obesity is well documented (621). Microgliosis in the MBH is a key feature of obesity, and attenuating microglia inflammatory activity can alleviate metabolic dysfunction during diet-induced obesity (149).

Alternatively, the role of microglia in modulating metabolic dysfunction during cachexia has not been investigated. The purpose of this study was to determine if microglia mediate cachexia during pancreatic cancer. We observed that both microgliosis and astrogliosis occur early in a mouse model of PDAC-associated cachexia. Surprisingly, we observed that microglia depletion worsened cachexia, including increased anorexia, muscle catabolism, and fatigue. We then showed, using an *ex vivo* culture system, that microglia respond to tumor-derived factors, producing large amounts of the transcript coding for arginase-1, a potently anti-inflammatory enzyme. These results suggest that microglia in the MBH protect against cachexia during PDAC.

2. Materials and Methods

2.1 Animals and CSF-1R antagonist treatment

Male 20–25g WT C57BL/6J (stock no. 000664) were purchased from Jackson Laboratories. Animals were aged between 7 and 12 weeks at the time of study, maintained at 27°C on a normal 12:12 hr light/dark cycle, and provided ad libitum access to water and food. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Oregon Health & Science University.

PLX5622 was generously provided by Parm Singh and Andrey Reymar at Plexxikon. This is an orally active CSF-1R antagonist previously shown to deplete microglia (622). PLX5622 was incorporated into chow by Research Diets. The same chow (AIN chow) without PLX5622 was provided as a control. Animals were placed on PLX5622-containing chow or AIN chow starting 6 days prior to tumor inoculation.

2.2 KPC Cancer Cachexia Model

Our lab generated a mouse model of PDAC-associated cachexia by a single injection of murine-derived KPC PDAC cells (originally provided by Dr. Elizabeth Jaffee from Johns Hopkins) (122). These cells are derived from tumors in C57BL/6 mice heterozygous for oncogenic KRAS^{G12D} and point mutant TP53^{R172H} (both downstream of lox-stop-lox cassettes) with expression induced and targeted to the pancreas via the PDX-1-Cre

driver (554). Cells were maintained in RPMI supplemented with 10% heat-inactivated FBS, and 50 U/mL penicillin/streptomycin (Gibco, Thermofisher), in incubators maintained at 37°C and 5% CO_2. In the week prior to tumor implantation, animals were transitioned to individual housing to acclimate to experimental conditions. Animal food intake and body weight were measured once daily. Sham-operated animals received PBS in the same volume. Voluntary home cage locomotor activity was measured via MiniMitter tracking devices (Respironics). Mice were implanted 6 days prior to tumor implantation with MiniMitter transponders in the intrascapular subcutaneous space. Using these devices, movement counts in *x*-axis, *y*-axis, and *z*-axis were recorded in 5 min intervals (Vital View, MiniMitter). Only dark cycle activity was analyzed, as our previous studies demonstrated little movement during the light phase (122).

Animals were euthanized between 7 and 10 days post inoculation, when food intake was consistently decreased and locomotor activity was visibly reduced, yet signs of end-stage disease (ascites, unkempt fur, hypotheremia, etc.) were not present (122).

2.2 Immunofluorescence immunohistochemistry

Mice were anesthetized using a ketamine/xylazine/acetapromide cocktail and sacrificed by transcardial perfusion fixation with 15 mL ice cold 0.01 M PBS followed by 25 mL 4% paraformaldehyde (PFA) in 0.01 M PBS. Brains were post-fixed in 4% PFA overnight at 4°C and cryoprotected in 20% sucrose for 24 hrs at 4°C before being stored at −80°C until used for immunohistochemistry. 30 µm free-floating sections were cut from perfused brains using a Leica sliding microtome. Sections were incubated for 30 min at

room temperature in blocking reagent (5% normal donkey serum in 0.01 M PBS and 0.3% Triton X-100). After the initial blocking step, sections were incubated in primary antibody in blocking reagent for 24 hrs at 4°C, followed by incubation in secondary antibody (also listed below) for 2 hrs at 4°C. Between each stage, sections were washed thoroughly with 0.01 M PBS. Sections were mounted onto gelatin-coated slides and coverslipped using Prolong Gold antifade media with DAPI (Thermofisher).

For primary antibodies, Rabbit anti-Iba-1 (Wako, NCNP24, 1:1000) and mouse anti-GFAP (Millipore, GA5, 1:1000) were used. The following secondary antibodies were used, all derived from donkey and purchased from Invitrogen, with dilution in parentheses: anti-rabbit AF488 (1:500), anti-mouse AF633 (1:500), and anti-rabbit AF555 (1:1000).

2.3 Image Acquisition and Analysis

All images were acquired using a Nikon confocal microscope. Three 7-layer flattened Z-stack images of the hippocampus, MBH, and cortex were acquired using a 20X objective. Images were 2048 x 2048 pixels, with a pixel size of 0.315 μm. The cortex was defined as the field of view immediately dorsal to the cingulum and directly lateral to the midline, also dorsal to the corpus collusum. The hippocampus was identified by the granule cell layer of the dentate gyrus, which was positioned at the left end of each image. The MBH was defined as the region surrounding the third ventricle at the base of the brain, starting rostrally at the end of the optic chiasm when the arcuate nucleus appears (−1.22 mm from bregma) and ending caudally at the mammillary body (−2.70 mm from bregma). Within the MBH, the arcuate nucleus and median eminence

were identified as shown in Chapter 3, Figure 3D. The arcuate nucleus was defined as the regions to the left and right of the third ventricle, and the median emenince was defined as the semicircular extension of tissue directly below the third ventricle.

Microglia and astrocyte morphology in the MBH and hippocampus were quantified using Fiji (ImageJ, NIH). Microglia morphology was also quantified in the cortex, but we did not perform astrocyte morphology analysis in this region due to lack of GFAP immunoreactivity in both sham and tumor groups. Images were uploaded by a blinded reviewer (KGB) and converted to 8-bit greyscale images. After thresholding, microglia and astrocytes were identified using the "analyze particle" function, which measured cell number (for microglia), mean GFAP intensity (for astrocytes), and percent area covered by Iba-1 or GFAP staining.

2.4 Quantitative real-time PCR

For in vivo experiments, mice were euthanized with a lethal dose of a ketamine/xylazine/acetapromide. Hypothalamic blocks were dissected, snap frozen, and stored in −80 °C until analysis. For both *in vivo* and *in vitro* experiments, RNA was extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was transcribed using TaqMan reverse transcription reagents and random hexamers according to the manufacturer's instructions. PCR reactions were run on an ABI 7300 (Applied Biosystems), using TaqMan universal PCR master mix with the following TaqMan mouse gene expression assays: *18s* (Mm04277571_s1), *Tnf* (Mm00443258_m1), *Il-6* (Mm01210732_g1)*, Il-1b* (Mm00434228_m1)*, Il-10*

(Mm01288386_m1), *Tgfb* (Mm01227699_m1), *Arg1* (MM00475988_m1), *Nos2* (Mm00440502_m1), *Tmem119* (Mm0052305_m1), and *Cd68* (Mm03047343_m1).

Relative expression was calculated using the ΔΔCt method and normalized to sham control. Statistical analysis was performed on the normally distributed ΔCt values.

2.5 Primary Microglia Culture and KPC-Conditioned Media Treatment

Primary mixed glial cultures containing microglia and astrocytes were prepared from neonatal mouse cortices. Brain cortices from 1 to 3-day old newborn mouse pups were dissected, freed of the meninges, and then digested with papain (Worthington Biochemical Corporation). The mixed cortical cells were passed through a 70µm cell strainer and seeded in 75cm^2 flasks in DMEM media (low glucose with L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin) Media was refreshed every 3-4 days for 9-16 days. Microglia were isolated by shaking flasks at 200 rpm at 37 °C for 1 hr. Cells were re-plated into 6 well plates at 5×10^5/well and maintained in DMEM media for 24 hrs before stimulation. More than 90% of these isolated cells were confirmed as microglia by Iba1 staining and flow cytometry (CD45+CD11b+ cells, data not shown).

KPC tumor cells were cultured in a 75-cm^2 flask until confluent. 24 hrs prior to treatment, 13 ml fresh media (RPMI supplemented with 10% FBS and 1% penicillin-streptomycin) was added for generating KPC-conditioned media. On the treatment day, 4 mL KPC-conditioned media mixed with 1 mL fresh RPMI media (to ensure treated microglia were not nutrient starved) was added to each of the three wells of the 6 well plate containing microglia. The other three wells each received 5 mL control media (RPMI media). Three additional wells received 5 mL control media containing 10 ng/ml

LPS. 16 hours after treatment, media was removed, and adherent cells were washed with PBS then lysed. RNA was then extracted using a Qiagen RNAEasy kit.

2.6 Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed with Prism 7.0 software (Graphpad Software Corp). When two groups were compared, data were analyzed with student's t-test. When more than two groups were compared, data were analyzed with One-way ANOVA analysis followed by Bonferroni *post hoc* analysis. For all analyses, significance was assigned at the level of $p < 0.05$.

3. Results

3.1 Hypothalamic microgliosis occurs early in PDAC cachexia

We first assessed whether microgliosis occurred in different brain regions during cachexia in our PDAC cachexia mouse model. This model is generated through a single intraperitoneal (IP) injection of C57BL/6 KRAS^{G12D} P53^{R172H} Pdx-Cre$^{+/+}$ (KPC) cells. This well-characterized model recapitulates all of the key signs and symptoms of cachexia seen in humans, including anorexia, muscle catabolism, fatigue, and loss of fat mass (122). We used Iba-1 to label brain macrophages, and assessed morphology and number at 7 days post inoculation (d.p.i.) (when the animals begin to develop muscle catabolism, anorexia, and fatigue) and 10 d.p.i. (when the animals were cachectic yet still a few days away from end-stage signs such ascites, hypothermia, etc.). We observed microglia accumulation during PDAC in the median eminence (ME) and arcuate nucleus (ARC) of the hypothalamus at 7 d.p.i. and continuing at 10 d.p.i. (Fig 1A-C). We also observed that microglia assumed an "activated" morphology, with retracted processes and increased soma size. This was evident in the ARC but was striking in the ME (insets in Fig 1A-C). Interestingly, microglia activation only occurred in the hypothalamus, with no increase in microglia number or Iba-1 immunoreactivity in the hippocampus or cortex throughout the course of PDAC cachexia (Fig 1A and E).

A

	Sham	7 days	10 days
Hypothalamus	Iba-1		
Hippocampus			
Cortex			
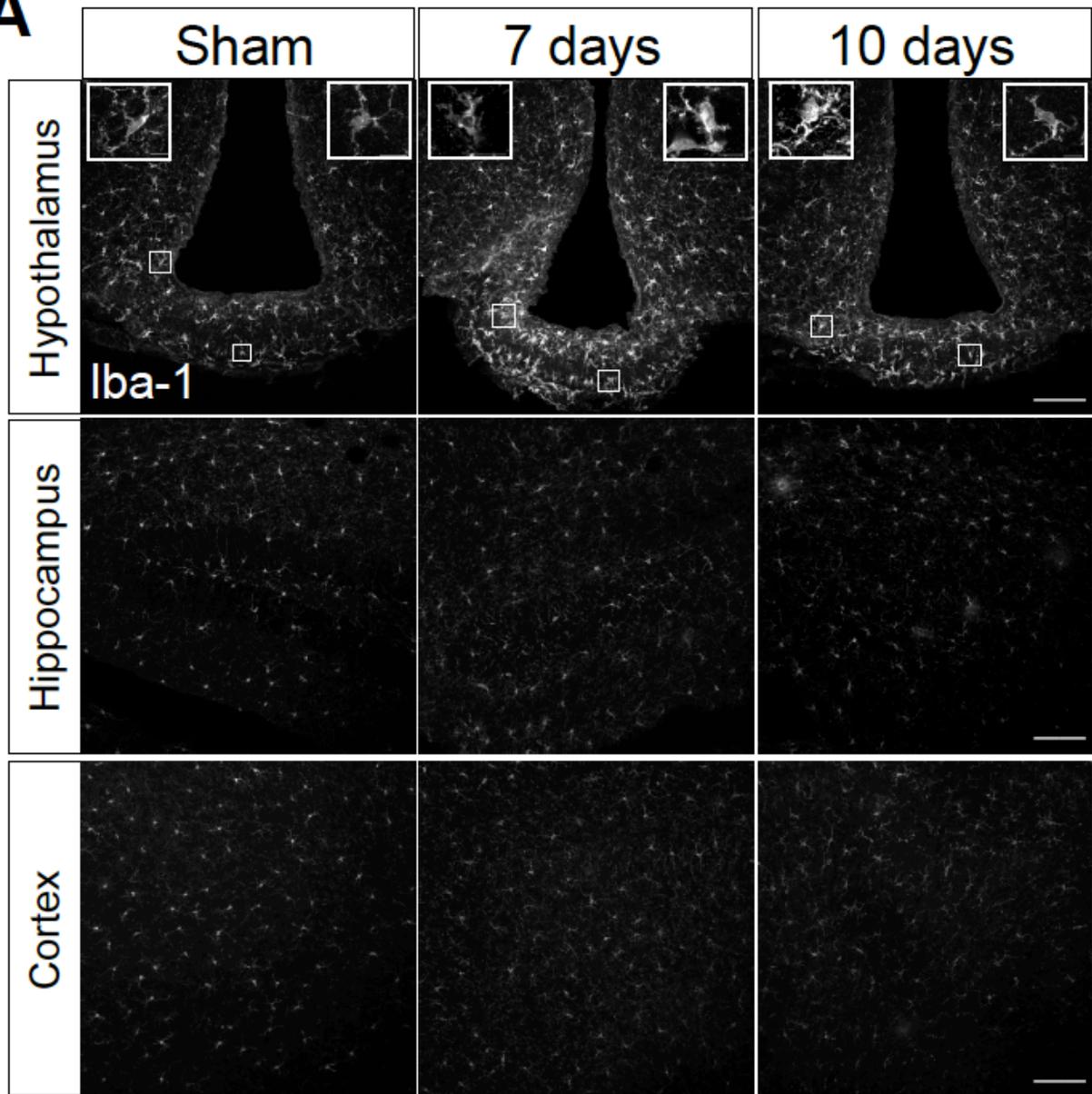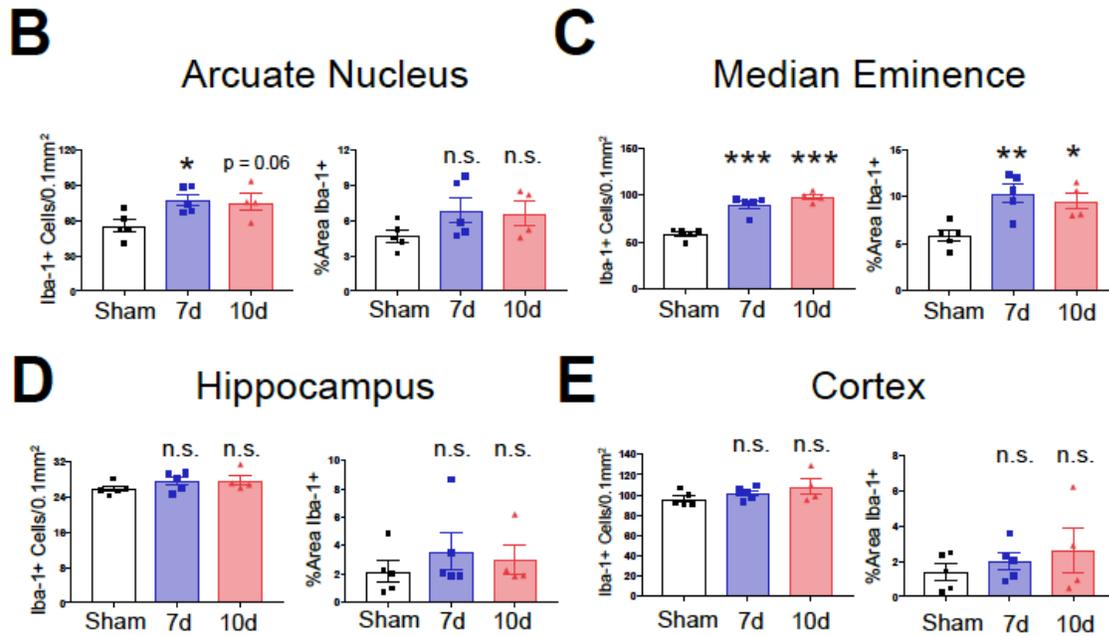
Figure 1. Microgliosis occurs in the MBH throughout PDAC cachexia. A) Representative confocal microscopy images of Iba-1 immunoreactivity within the MBH, hippocampus, and cortex from either a sham, 7 d.p.i., or 10 d.p.i. mouse brain. Scale bar = 100 μm. Top left inset = ARC microglia within left box. Top right inset = ME microglia within right box. Inset scale bars = 10 μm. B-E) Analysis of microglia number (left) or percent area Iba-1+ (right) in the arcuate nucleus, median eminence, hippocampus, or cortex. n.s. = not significant, ***$P<0.001$, **$P<0.01$, *$P<0.05$ compared to sham in one-way ANOVA analysis. For all figures, bars depict mean ± s.e.m.

3.1 Astrogliosis occurs early in PDAC cachexia

Next, we assessed whether astrocytes assumed an activated morphology during PDAC cachexia. Using the same experimental setup described above for microglia, we used GFAP labeling to analyze astrocyte morphology in the hippocampus and hypothalamus at 7 and 10 d.p.i. We were unable to detect astrocytes within the cortex and it was

therefore excluded from analysis. Similar to our findings for microglia, we observed astrocytosis in the MBH beginning at 7 d.p.i. and becoming even more most robust at 10 d.p.i. (Fig 2A-C) Unlike our findings for microglia, however, astrogliosis was just as robust in the hippocampus, with mean GFAP fluorescent intensity increased at 7 and 10 d.p.i. (Fig 2A and D) While percent area covered by GFAP was not significantly increased in the hippocampus at either 7 or 10 d.p.i., a trend was observable (Fig 2D).
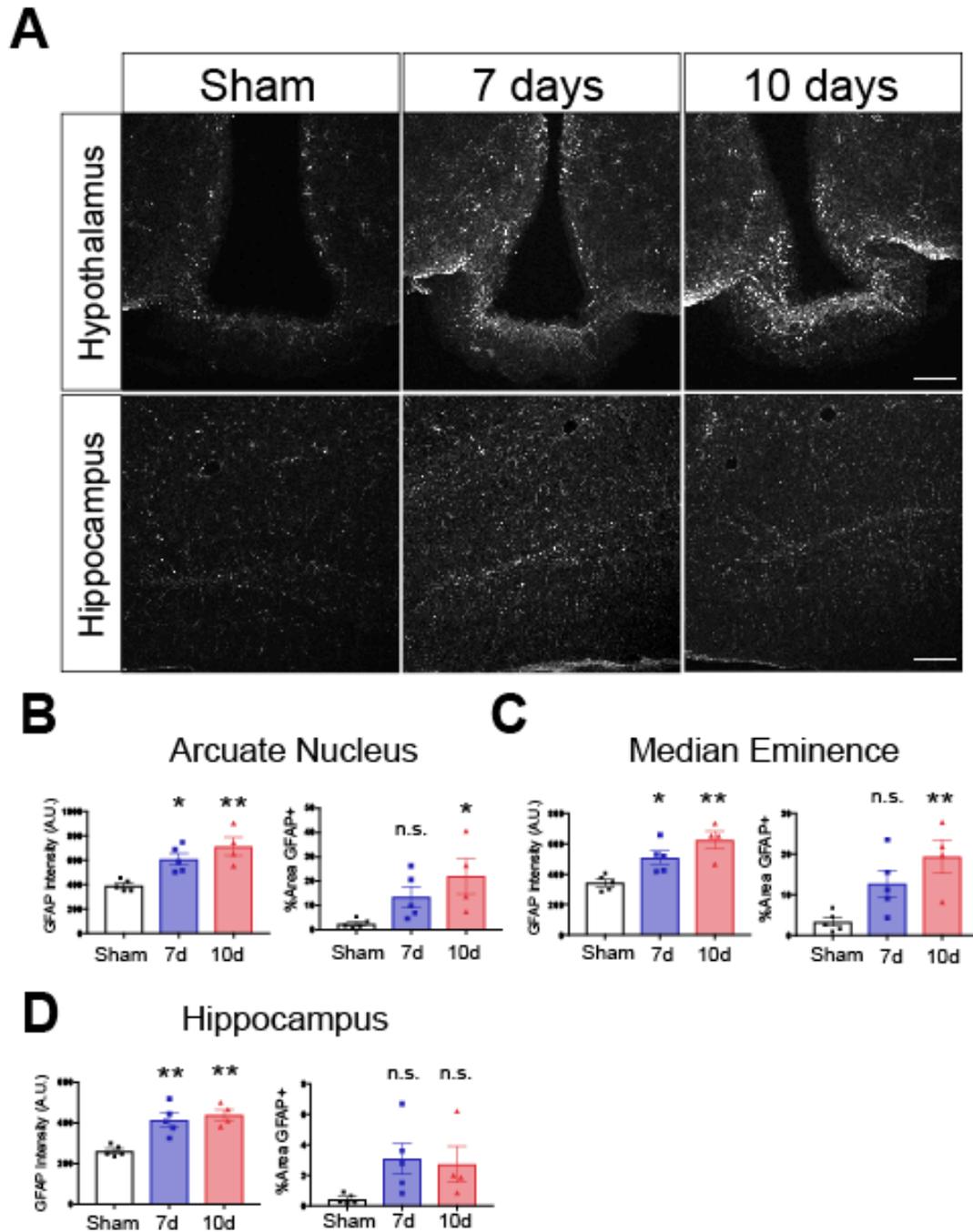
Figure 2. Astrogliosis occurs in the MBH and the hippocampus throughout PDAC cachexia. A) Representative confocal microscopy images of GFAP immunoreactivity within the MBH and hippocampus from either a sham, 7 d.p.i., or 10 d.p.i. mouse brain. Scale bar = 100 μm. B-D) Analysis of GFAP fluorescent intensity (left) or percent area

GFAP+ (right) in the arcuate nucleus, median eminence, or cortex. n.s. = not significant, **P<0.01, *P<0.05 compared to sham in one-way ANOVA analysis.

3.3 Microglia depletion worsens PDAC cachexia

Based on our observation that PDAC induces microglia activation in a region important for appetite, activity, and body composition, we hypothesized that microglia influence cachexia symptoms. To test this hypothesis, we utilized an oral CSF1R inhibitor developed by Plexikkon (graciously provided as a gift by Dr. Parm Singh), which depletes 75% of microglia within 3 days of administration, and 99% by 7 days (622). We conducted three independent studies in which we treated animals with PLX5622-containing chow or control for 6 days then inoculated animals with KPC tumor cells IP. Animals were maintained on PLX5622 or control chow for the duration of the study, and euthanized at either 7, 9, or 10 d.p.i. (Fig 3A). In the 6 days prior to tumor inoculation, PLX5622 did not induce any changes in food intake, body weight, or locomotor activity (data not shown). Interestingly, we observed that microglia depletion worsened cachexia, as evidenced by the fact that tumor-bearing animals treated with PLX5622 experienced increased anorexia compared to untreated tumor-bearing animals, beginning at 6 d.p.i. (Fig 3B and C). There was no difference in food intake between groups prior to cachexia development (first 5 days, Fig 3C). PLX5622 treatment also was associated with decreased home cage locomotor activity in tumor-bearing animals compared to untreated animals during the cachexia stage (final four days of the study, Fig 3D and E). When we compared muscle catabolism, we observed a similar trend, in

that in all three studies tumor-bearing animals treated with PLX5622 had decreased

gastrocnemius mass compared to that of untreated tumor-bearing animals (Fig 3F).
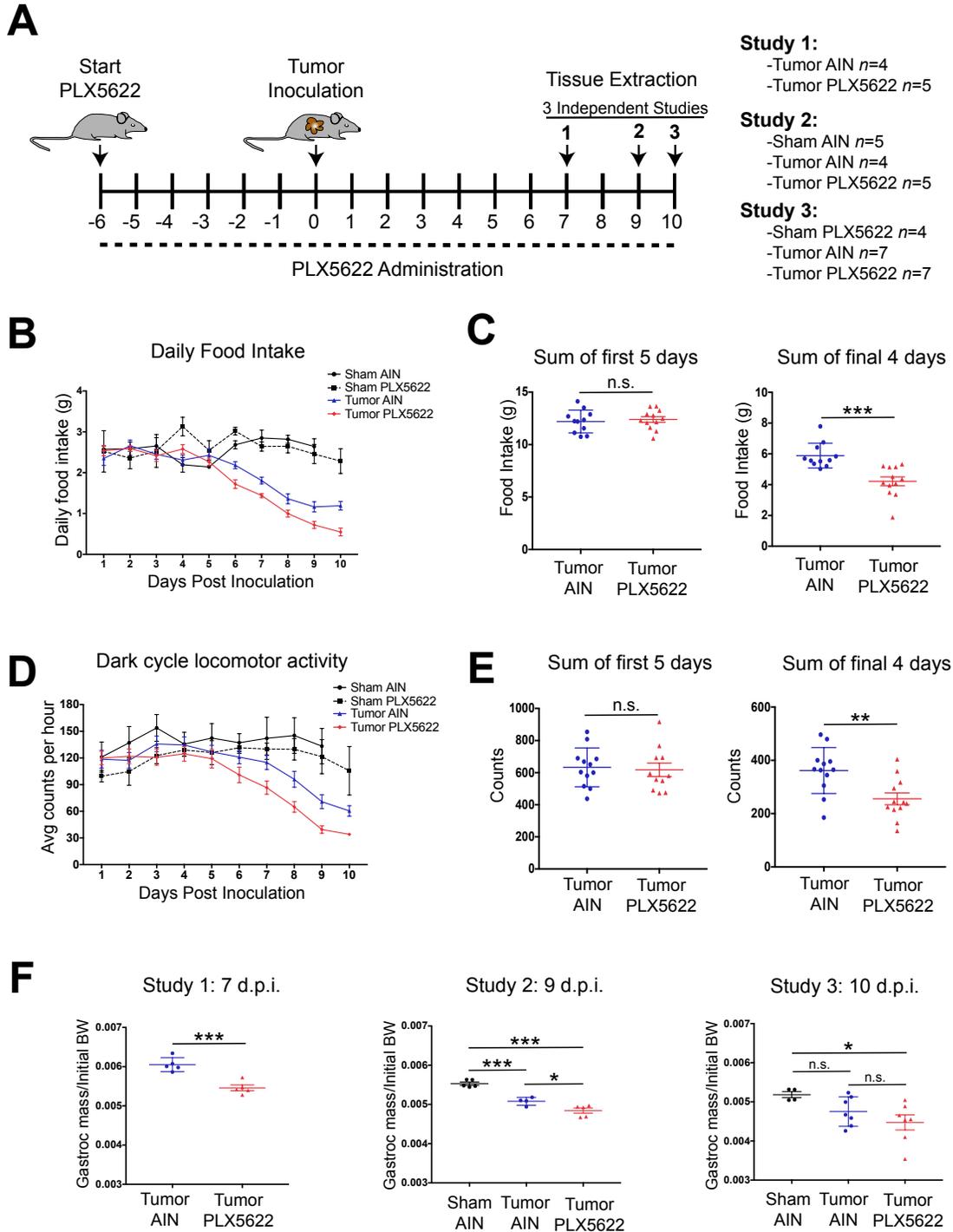
Figure 3. Microglia depletion worsens PDAC cachexia. A) Schematic of PLX5622 treatment protocol and tumor inoculation. Diagram depicts three independent studies investigating various stages of cachexia. Numbers listed on number line are in relation to tumor inoculation. Day 0 = tumor inoculation. B) Daily food intake after tumor inoculation, with two sham groups shown for reference. AIN = control chow. For food intake analysis, studies 2 and 3 were combined. C) Total food intake during the first 5 days of the study (pre-cachexia stage) and final 4 days of the study (cachexia stage). n.s. = not significant, ***P<0.001 in student's t-test. D) Dark cycle home cage locomotor activity after tumor inoculation, with two sham groups shown for reference. Data shown are study 2 and 3 pooled. E) Sum of average hourly counts for the first 5 days of the study (pre-cachexia stage) and final 4 days of the study (cachexia stage). n.s. = not significant, **P<0.01 in student's t-test. F) Muscle catabolism, determined by gastrocnemius mass normalized to initial body weight. Studies were not pooled since normalized muscle mass varied between study, likely due to effects of paraformaldehyde perfusion. n.s. = not significant. For study 1 (left panel), ***P<0.001 in student's t-test. For studies 2 and 3, ***P<0.001, *P<0.05 in one-way ANOVA analysis.

We verified microglia depletion with Iba-1 immunofluorescence, which showed greater than 90% microglia depletion in both tumor-bearing and non-tumor-bearing animals. Interestingly, we did observe an increase in number of microglia in the PLX5622-treated tumor animals compared to the PLX5622-treated sham animals. However, this was still far less than the number observed in the untreated animals (Fig 4).
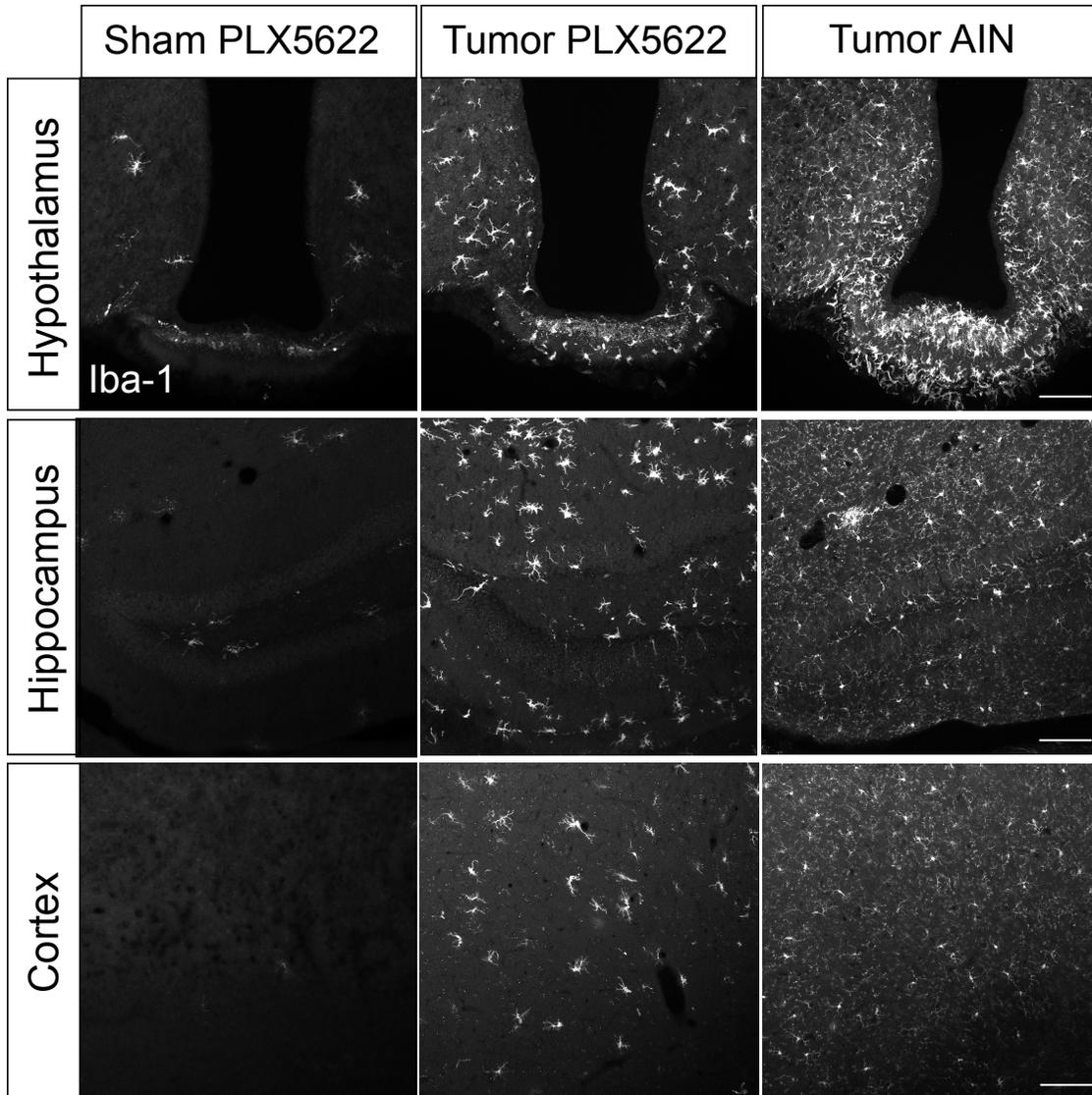
Figure 4. PLX5622 depletes microglia. Iba-1 immunolabeling to detect microglia in animals treated with PLX5622 chow to deplete microglia or AIN control chow. All groups are after 16 days on respective chow. Tumor groups euthanized at 10 d.p.i. Scale bar = 100 um.

3.4 Microglia respond to PDAC tumor-derived factors and produce arginase-1 both *in vivo* and *ex vivo*.

Since microglia depletion worsened anorexia, muscle catabolism, and fatigue during PDAC, we hypothesized that these cells provide protection against cachexia. In order to identify possible "protective" factors produced by microglia in response to PDAC we utilized an *ex vivo* culture system in which isolated primary microglia were exposed to KPC-conditioned media. We isolated mixed glia from one to three-day old mouse pups, then after 14-16 days we removed microglia through shaking and treated them with KPC-conditioned media (Fig 5A). Compared to the positive control (10 ng lipopolysaccharide [LPS]), we observed minimal upregulation of the pro-inflammatory transcripts *Il-1b*, *Tnf*, *IL-6*, and *Nos2* by KPC conditioned media (Fig 5B). Alternatively, we observed robust upregulation of the anti-inflammatory transcript *Arg1* (coding for the enzyme arginase 1). While LPS induced a substantial upregulation of the anti-inflammatory cytokine *Il-10* (465-fold increase)*,* KPC conditioned media treatment resulted in massive downregulation of this transcript (40-fold decrease). Neither LPS nor KPC-conditioned media caused any change in expression of the anti-inflammatory transcript *Tgfb*. Lastly, we assessed expression of transcripts previously identified as markers microglia activation (623). We observed a robust decrease in the transcript *Tmem119* in both LPS- and KPC conditioned media-treated microglia (indicative of microglia activation) (Fig 5B).

We next assessed whether these transcripts were differentially regulated during PDAC *in vivo*. We dissected hypothalamic blocks from tumor-bearing and sham animals at 10 d.p.i. and extracted RNA for qRT-PCR. Similar to what occurred in KPC-conditioned media treated primary microglia, we observed increased expression of *Il-1b* and *Arg1* in the hypothalamii of tumor-bearing animals compared to sham animals,

whereas there was decreased expression of *Nos2* and *Tmem119* (Fig 5C). There was no change in *Tnf*, *Il-6*, *Il-10*, or *Cd68* in PDAC mice.
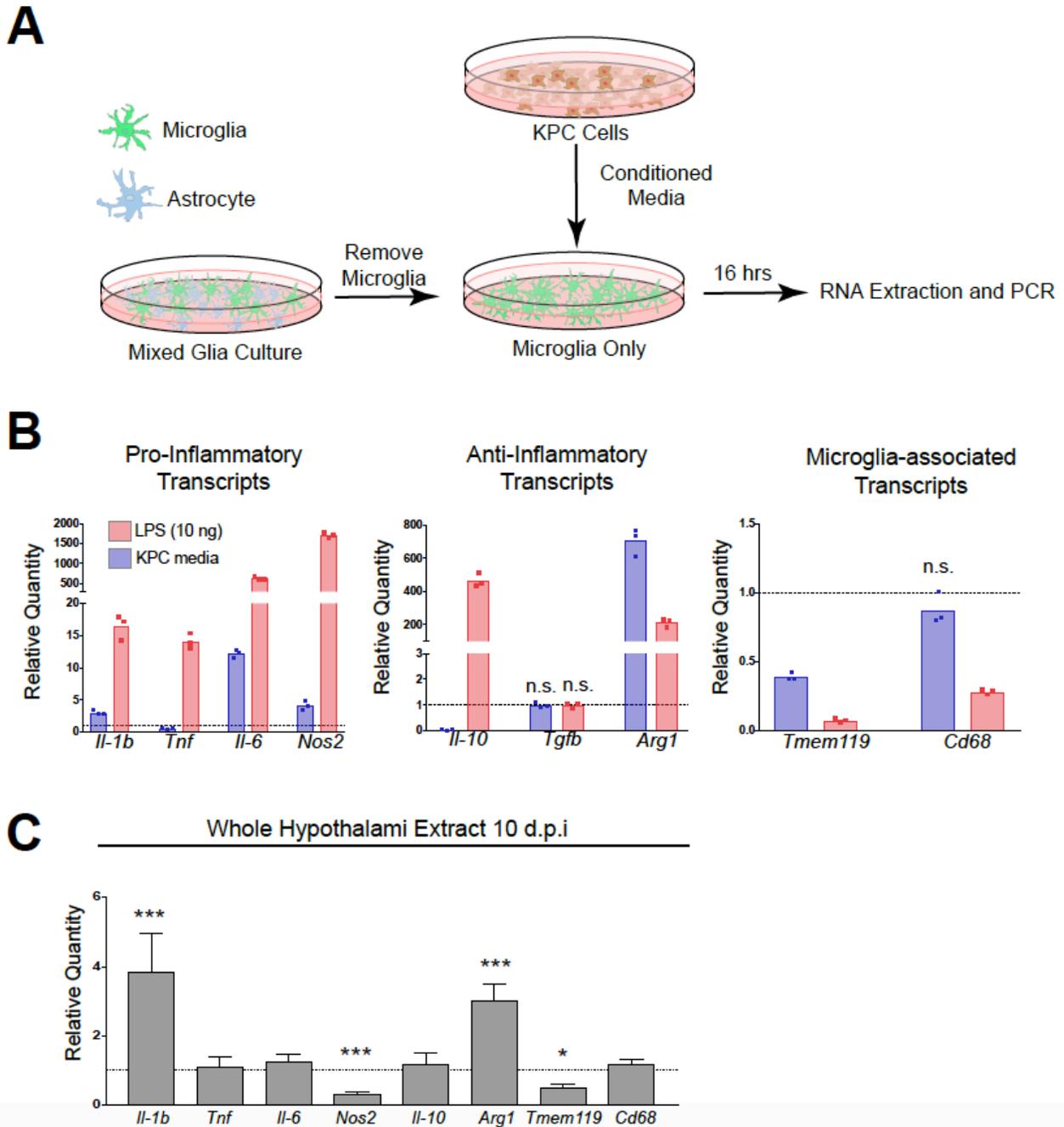
Figure 5. PDAC induces inflammatory mediator expression in *vitro* and *in vivo*. A) Schematic representation of *ex vivo* KPC-conditioned media treatment system. B) qRT-PCR analysis of pro-inflammatory, anti-inflammatory, and microglia-associated

transcript from primary microglia treated with either KPC-conditioned media or 10 ng LPS. Values are relative to those from control media-treated primary microglia. All comparisons at least *P*<0.01 compared to control media-treated in repeated measures one-way ANOVA unless indicated otherwise. n.s. = not significant. Results are representative of two independent experiments. C) qRT-PCR analysis of the same transcripts as B (except *Tgfb*) in RNA extracted from whole hypothalamii. Results are relative to sham. ***P<0.001, *P<0.05 relative to sham in repeated measures one-way ANOVA. Results consist of two independent experiments combined.

4. Discussion

In summary, we observed gliosis within the MBH throughout the course of PDAC cachexia. Microgliosis was specific to the MBH, while astrogliosis also occurred in the hippocampus. Microglia depletion with an oral CSF-1R antagonist worsened cachexia, including increased anorexia, fatigue, and muscle catabolism. PDAC-derived factors induced microglia to produce arginase-1, a potently anti-inflammatory enzyme. Contrary to previous studies suggesting that microglia activation is detrimental in disease processes, these results implicate microglia as neuroprotective during pancreatic cancer cachexia.

We demonstrated that microglia in the MBH display an activated morphology early in PDAC cachexia, which correlated with astrogliosis in this region. Microgliosis peaked at 7 days post-inoculation, a time point when animals were only beginning to develop anorexia and muscle catabolism. Alternatively, astrogliosis did not peak until 10 days post-inoculation, a time when animals were profoundly anorexic, lethargic, and catabolic, yet were still a few days away from showing terminal signs (ascites, hypothermia, lack of grooming, etc.). This is the first study to show that microgliosis occurs during cancer cachexia. While reviews hypothesized that microglia activation may contribute to hypothalamic inflammation and subsequent symptoms of cancer cachexia (624), this was the first study to directly test this hypothesis. We observed that microglia assumed morphologies that resembled "activated microglia", with retracted processes and increased soma size. One study by Norden et al., assessing the role of microglia in cancer-associated fatigue and depression, reported that microglia in the cortex, but not the hippocampus, displayed an activated phenotype three weeks after

inoculation with the colon-26 tumor model (625). While we also did not observe microglia activation in the hippocampus, our results conflict with those of Norden et al. in that we did not detect any differences in cortex microglia morphology in our PDAC tumor model. This discrepancy may be due to the tumor line (colon-26 vs. KPC), mode of inoculation (subcutaneous vs. IP), or cancer stage (21 d.p.i. vs. 7 d.p.i.). Moreover, the authors did not report hypothalamic microglia morphology, which may well have exhibited activated morphology. Further studies are needed to assess the differences in microglia activation status throughout the brain in different tumor lines.

We observed regional differences in microglia morphology throughout the brain, and even between different hypothalamic nuclei. While there were no differences in microglia morphology in the hippocampus and cortex of tumor-bearing animals compared to sham animals, activated microglia were noticeable in the arcuate nucleus of tumor-bearing animals as early as 7 d.p.i. Microglia activation was even more robust in the median eminence in tumor-bearing animals. Studies investigating microglia activation in the context of high fat diet-induced obesity (HFDO) showed similar results in that microglia in the MBH exhibited a much more robustly activated phenotype than in other brain regions during HFDO (172). These results are likely due to the ME lacking a BBB and therefore providing free access to circulating factors. We chose to limit analysis complexity and did not include other circumventricular organs, but it is likely that macrophages in regions such as the area postrema and subfornical organ display an activated morphology during PDAC. Since the ME contains terminals of secretory neurons important in the hypothalamic-pituitary-adrenal axis, future studies should

assess whether resident macrophages are capable of modulating HPA axis activity during cancer.

We observed an increased number of microglia, both in the arcuate nucleus and median eminence, early in PDAC cachexia. While many studies show that microglia accumulate in the MBH during HFDO (149, 172, 292), this is first study to show this phenomenon during a peripheral cancer. As described in Chapter 4, we showed that peripheral macrophages accumulated in the ME during PDAC, but did not appear to infiltrate into the parenchyma. This suggests that microglia accumulation within the ME and arcuate nucleus was due to cell division, a phenomenon that also occurs during HFDO (172, 292, 626). We are currently conducting studies to confirm microglia division in the MBH during PDAC (using Ki-67 and BrdU labeling).

Based on the hypothalamic microglia morphology we observed, indicative of an "activated status", we hypothesized that microglia depletion would attenuate cachexia. Surprisingly, microglia depletion worsened PDAC cachexia, suggesting that microglia are neuroprotective during pancreatic cancer. Our results are agreement with a growing amount of literature showing that microglia activation is neuroprotective in a variety of contexts, including stroke (627), Alzheimer's disease (628), and multiple sclerosis (629). Alternatively, our results differ from studies on anxiety, which demonstrated that microglia activation is associated with anxiety-induced impaired social interaction, which the authors used as a measure of "sickness behaviors" (630). In contrast, we observed greatly decreased home cage locomotor activity in animals treated with PLX5622. These discrepancies suggest that microglia response to stimuli differs depending on context, and that inferring function based on morphology is simply not possible. This is

further emphasized by the fact that, similar to PDAC cachexia, microglia assume an activated state in the MBH in HFDO, during which there is also induction of inflammatory cytokine transcripts. However, PLX5622 treatment ameliorated certain disease aspects during HFDO (149), while we observed that PLX5622 treatment worsened all cachexia aspects measured. It is challenging to directly compare HFDO to PDAC cachexia, yet we observed that KPC-conditioned media induces a completely different transcriptional response in microglia than that which was reported to be induced by saturated fatty acids (631).

We demonstrated, using an *ex vivo* culture system, that tumor-derived factors stimulate microglia to produce arginase-1 (Arg1), a peptidase that cleaves L-arginine into urea and ornithine. PDAC is a powerful inducer of Arg1 in macrophages, likely from tumor-derived lactate (632). Arg1 is a potently anti-inflammatory enzyme that is thought to modulate inflammation through directly competing with nitric oxide (NO) synthase for arginine (633). NO is a cytotoxic gaseous free radical that can cause neuronal damage and alter activity (634). We are currently conducting histology studies to assess for microglial Arg1 expression *in vivo*, as well as markers of neuropathology in PLX5622-treated tumor-bearing animals (BBB breakdown, endoplasmic reticulum stress, reactive oxygen species generation, etc.)

For microglia and other brain macrophages, Arg1 is commonly used as a polarization marker, indicating an "anti-inflammatory" or "M2" phenotype (623). However, the function of resident macrophage-derived Arg1 in the brain is unclear, and we believe judging function from polarization status is a gross oversimplification that can result in overlooking key functional features (635). For example, we observed a robust decrease

in *Il-10* and *Tgfb* transcript in KPC-conditioned media treated primary microglia. Both of these transcripts are frequently implicated as important "M2" transcripts. Our data show that during PDAC microglia function is far more complicated than a simple "pro-inflammatory" or "anti-inflammatory" phenotype. These results add to the growing amount of evidence demonstrating that microglia polarization is a useless measure that should be discontinued.

Several limitations should be considered when interpreting the results of the present study. We administered a systemic CSF-1R antagonist, which may have exerted effects on macrophages outside the brain. Previous studies show that prolonged PLX5622 exposure can deplete tumor-associated macrophages, but does not affect other macrophages populations such as those in the spleen or marrow (636). We cannot rule out that the effects of PLX5622 treatment we observed were not due to tumor-associated macrophage depletion. Furthermore, we observed an increased number of microglia in the brains of PLX5622-treated tumor animals compared to PLX5622-treated sham animals. These results may be due to the decreased food intake of the tumor animals, which could result in decreased drug dose. This in turn may have allowed for microglia repopulation and influenced results. However, we recently completed a study in which sham animals were pair-fed the same amount of PLX5622 chow as PLX5622-treated tumor animals, and preliminary observations show that sham pair-fed PLX5622 treated animals had nearly the same number of microglia as sham animals that were given PLX5622 chow *ad libitum*. This suggests that tumor-associated factors may cause microglia division in PLX5622-treated tumor animals. We are currently performing flow cytometry and histology studies to verify these results, as well

as determine the degree of macrophage depletion in other tissues after PLX5622 treatment.

In conclusion, microglia respond to tumor-derived factors and assume an activated state in the MBH during PDAC. Depleting microglia or preventing their activation worsens cachexia, demonstrating their protective role during pancreatic cancer. Further studies are needed to identify the key protective molecular mediator presumable produced by these cells.

Acknowledgements

Summary and Conclusions

Cachexia is a devastating state of malnutrition characterized by appetite loss, tissue catabolism, and fatigue. Although this syndrome occurs during many chronic diseases, this dissertation focused on pancreatic ductal adenocarcinoma (PDAC), a deadly malignancy of the exocrine pancreas frequently accompanied by cachexia. Neuroinflammation is a key component of cachexia syndrome, yet cellular sources of inflammatory molecules in the brain are not known. This dissertation focused on myeloid cells in the brain as neuroinflammatory drivers of cachexia during pancreatic cancer. Both brain-resident macrophages and brain-infiltrating leukocytes were investigated.

Chapter 1 provided an extensive literature review, which demonstrated a role for hypothalamic inflammation in the pathogenesis of cancer cachexia. Several cytokines that can act directly on neuronal populations in the hypothalamus were implicated, such as Il-1β, IL-6 and TNF-α. The literature review revealed that no studies identified cellular sources of these molecules during cachexia. Infiltrating immune cells were suggested as potential sources that should be investigated. Previous studies of chronic viral infection and inflammatory liver disease demonstrated that molecules associated with infiltrating immune cells, such as chemokines and leukocyte adhesion molecules, are induced in the brain during chronic systemic inflammation. This suggests that the machinery is in place for immune cell infiltration into the brain, yet no studies directly assessed for the presence of brain-infiltrating immune cells during cancer cachexia.

Chapter 2 is a literature review on hypothalamic dysfunction in multiple sclerosis (MS). Fatigue and weight dysregulation are common in MS, yet treatments for these

symptoms are lacking, mainly due to the fact that mechanisms are not well understood. This disease is driven by brain-infiltrating T-cells and therefore provides a potential avenue for studying mechanisms and functions of infiltrating immune cells in the brain. Since wakefulness and body composition are controlled by the hypothalamus, we hypothesized that hypothalamic dysfunction, possibly regulated by brain-infiltrating leukocytes, drives fatigue and weight dysregulation during MS. Literature review revealed that studies investigating such mechanisms are sparse, but demonstrate a potential role for cortisol and/or orexin dysregulation. Therefore, future studies focusing on the hypothalamus during MS are badly needed. This chapter presented insights from MS studies that can be applied to cancer cachexia studies.

Chapter 3 presented investigations into the molecular mechanisms of cachexia. This chapter first demonstrated that TRIF, a poorly studied adaptor protein key for innate immune activation, is important for hypothalamic inflammation and sickness behaviors after TLR4 stimulation. It then showed that TRIF is a key mediator of cancer cachexia, demonstrated by the fact that TRIF deletion resulted in attenuated anorexia, fatigue, neuroinflammation, and muscle catabolism during PDAC. This chapter set the stage for Chapters 4 and 5 by showing that while it is important to identify molecular mediators, cellular mediators must be determined to fully understand mechanisms of cachexia and eventually develop therapeutic strategies.

Chapter 4 presented extensive investigations into cellular mechanisms of cancer cachexia. In this chapter, it was demonstrated that myeloid cells infiltrate the brain early in a mouse model of PDAC cachexia. The majority of these cells were neutrophils, which accumulated at a unique CNS interface called the velum interpositum. This

implicated the velum interpositum as a novel gateway for immune cells to access the brain from the periphery during chronic systemic inflammation. Interestingly, a large percentage of neutrophils in this region expressed CCR2, which is typically considered a monocyte chemotaxis receptor. CCR2 deletion attenuated cachexia and prevented neutrophils from infiltrating the velum interpositum during PDAC. There were no differences in liver- or tumor-infiltrating neutrophils in CCR2 knockout tumor animals compared to WT tumor animals, showing that CCR2 is important specifically for neutrophil recruitment to the brain. This genetic approach was supplemented with a pharmacologic approach involving blockade of brain macrophage purinergic receptor signaling by intracerebroventricular administration of oxidized ATP. Oxidized ATP treatment in the CNS prevented neutrophil recruitment to the brain and attenuated cachexia. Lastly, Chapter 4 demonstrated that during PDAC brain-infiltrating neutrophils express a transcriptome that is distinct from that of neutrophils in the liver, tumor, and circulation. This suggests that a distinct neutrophil population infiltrates the brain during PDAC.

Several questions arise from studies described in Chapter 4. What molecules produced by neutrophils cause cachexia? Do these molecules act directly on neurons to mediate their activity? This could be addressed by injecting different neutrophil-derived enzymes and inflammatory molecules (neutrophil elastase, myeloperoxidase, neutrophil granule protein, etc.) directly into the brain and then assessing whether they activate neuron populations (via c-Fos labeling) in the MBH and cause sickness behaviors. For example, transthyretin, which was highly expressed specifically by brain-infiltrating neutrophils, was previously reported to induce anorexia and weight loss in rats (637).

Another plausible mechanism by which brain-infiltrating neutrophils induce cachexia is by causing neuropathology (neuronal degeneration, reactive oxygen species, blood brain barrier breakdown, etc.). This was not addressed during this dissertation as it has yet to be established whether neuropathology occurs in cachexia. A new graduate student in our lab is currently investigating markers of neuropathology during PDAC cachexia. Future studies could also involve deleting certain enzymes and inflammatory mediators from neutrophils (using the MRP8[Cre] mouse, which expresses a neutrophil-specific Cre recombinase) and assessing whether this results in attenuated or abrogated cachexia. Lastly, while CCL2 and CCR2 were the main focus, other chemokines such as CXCL1 and CXCL2 should be assessed as well.

Chapter 5 contains studies investigating the role of microglia in PDAC cachexia. It showed that during PDAC microglia accumulated in the MBH and became activated. Surprisingly, microglia depletion worsened cachexia during PDAC. Chapter 5 also showed that microglia respond to tumor-derived factors by expressing high levels of *Arg1*, which codes for arginase-1, a potently anti-inflammatory enzyme. Increased levels of *Arg1* were also evident in the hypothalamus during PDAC cachexia. These results lead us to hypothesize that microglia are protective against cachexia during PDAC. Several follow-up studies are needed to test this hypothesis. First, blocking arginase-1 in the brain (there are commercially available small molecule inhibitors) during PDAC and assessing whether this results in worsened cachexia would help support our hypothesis. Alternatively, adding arginase-1 to the brain during PDAC and assessing whether this results in attenuated cachexia would also support this hypothesis. This would be much more challenging than blocking arginase-1, since

recombinant enzymes are always difficult to work with *in vivo*. RNASeq studies on microglia isolated from hypothalamii throughout the course of PDAC would help identify additional "protective factors" produced by these cells. Once additional protective factors are identified, they could be deleted from microglia (if a mouse line with the associated allele floxed is available) with the tamoxifen-inducible fractalkine Cre mouse line (638). Then we could assess whether these animals had worsened cachexia. Obviously, such studies would be expensive and time-consuming. However, they would provide substantial mechanistic insights.

In conclusion, this dissertation identified a unique population of brain-infiltrating neutrophils that possibly mediates PDAC cachexia. It provided differing roles for resident immune cells ("protective") and infiltrating immune cells ("harmful") during cachexia. This work presents a novel organ-specific immune cell recruitment mechanism that occurs during chronic systemic inflammation. While follow-up studies are needed to verify these results, they provide potential therapeutic targets at both the cellular (neutrophils) and molecular (CCR2/CCL2) level for a condition that desperately needs treatment options.

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