Meningeal Lymphatic Vessels Contribute to Microglia Activation and Behavioral Response to Peripheral Inflammation

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Chapter 1 – Introduction

Throughout history, disease has been recognized as a looming threat to humanity however, prior to the 19th century, we understood very little about the causes of sickness and falling ill could only be attributed to divine punishment or cryptic natural phenomena (Atkins, 1984). Concurrent with the rise of germ theory, our understanding of what it means to be sick expanded to include the host response as well as the actions of the invader.

Sickness triggers both homeostatic and behavioral changes to promote survival yet the behavioral aspects have been comparatively understudied. The disparity in our understanding of the behavioral and homeostatic changes that occur in sickness may be in part due to the traditional dogma of the central nervous system (CNS) as an immune privileged zone which minimizes the importance of communication between the immune system and the CNS (Dantzer, 2018). The first behavioral effects of cytokine signaling, a broad category of cellular signaling molecules known primarily for their role in the immune response, in the CNS were described in the 1980s when interleukin-1 (IL-1) was found to influence sleep, and TNFα was found to influence appetite (Krueger et al, 1984; Plata-Salamán et al, 1988). Recent findings in the field of neuroimmunology further challenge the notion of CNS isolation from the immune system and have accelerated interest in understanding behavioral changes as a component of the immune response. In 2015, lymphatic vessels were discovered within the meninges that effect cognition, and drain fluid and immune cells from the CNS, a feature thought to be absent from immune privileged organs (Da Mesquita et al, 2018b; Louveau et al, 2015; Ma et al, 2017). In 2017, a neuromodulatory role for the cytokine IL-17 was discovered in *C. elegans*, marking the first definitive report of a cytokine in a non-immunological role, directly modulating the behavioral response to a stimulus (Chen et al, 2017) and now a growing body of evidence

implicates maternal immune activation and aberrant immunological profiles in the pathogenesis of several neurodevelopmental disorders (Hsiao et al, 2012; Kim et al, 2017).

This thesis is the summation of my studies into the mechanisms by which inflammatory signals originating outside of the CNS ultimately induce cellular changes within the brain and behavioral changes at the organismal level. In the following chapter, I will introduce the concept of the behavioral component of the innate immune response and provide an overview of research into one of the most well-studied initiators of this behavioral response, the cytokine interleukin 1β (IL-1β). Furthermore, I will summarize established and theorized routes of signal transduction from the immune system to the brain and highlight recent findings regarding lymphatic vessels located in the meninges and how this structure may facilitate a brain-immune interface. Lastly, I will summarize these findings and lines of evidence in the rationale for my thesis studies.

1.1 The Behavioral Component of the Immune Response

The study of sickness behavior can trace its roots back to the late 19th century when physician William Osler noted that a reduction of mental clarity and elevated apathy may be considered as symptoms of infection (Osler & Cousland, 1999) however, the earliest definitive report of infection-induced behavioral changes that promote survival was published in *Science* in 1975 in which researchers used bacterially infected lizards (*Dipsosaurus dorsalis*) to confirm the adaptive value of fever, which was controversial at the time. As ectotherms, reptiles must regulate their body temperature by seeking external heat sources and in the context of infection, they will induce fever through behavioral means therefore, fever can be controlled without the need for potentially confounding pharmacological agents used to investigate fever in mammals. The report concludes that an increase in body temperature substantially improves survival, establishing the existence of infection-induced behavior changes that contribute to the immune response and promote survival (Kluger et al, 1975). Nearly a decade later in 1984, James Krueger et al. recorded an increase in short wave sleep duration in rabbits following IL-1β injection, marking the first documented behavioral component of the mammalian immune response. In his report, Krueger emphasizes his finding that the sleep-inducing properties of IL-1 are distinct from its pyrogenic or fever-inducing activities as one can be ablated without effecting the other (Krueger et al, 1984). We now know that cytokines IL-1, IL-6, and TNFα, each possess the capability to induce the full suite of 'sickness behavior' symptoms (Kaster et al, 2012; Knoll et al, 2017; Sukoff Rizzo et al, 2012). These findings served to solidify the notion that behavioral changes in response to pathogens were not just by-products of illness but part of the host immune response itself, laying the groundwork for further characterization of the behavioral component of the immune response.

In the ensuing decades, the set of behavioral responses to immune activation have been termed ‘sickness behavior’ and expanded to include: lethargy, anhedonia, increased anxiety-like behavior, reduced self-grooming, and a reduction in social and sexual activities (Dantzer, 2001). It should be noted however, that the characteristic decrease in overall locomotor activity is a confounding factor that makes characterization of additional symptoms problematic, a fact that is typically acknowledged in reports which make claims about aspects such as anxiety-like behavior (Swiergiel & Dunn, 2007). Regardless of the difficulty inherent in parsing apart and characterizing facets of this response, roughly equivalent expressions of sickness behavior have been described in diverse vertebrate species including birds, fish, and mammals, indicating high adaptive value, thus emphasizing the importance of understanding this response (Kirsten et al, 2018; Tazi et al, 1988; Wilsterman et al, 2020). The study of sickness behavior may have significant clinical relevance as researchers have long noted the high overlap between mood disorder and sickness behavior symptoms, leading to the use of peripheral immune stimulation as a standard model of depressive behavior (Lasselin et al, 2020; Müller & Ackenheil, 1998; Zhao et al, 2020). In fact, depression symptoms have been identified as a side effect of cytokine based therapies developed for non-psychiatric pathology and recent meta-analysis of clinical studies show that an inflammatory profile can predispose patients to mood disorders (Capuron & Miller, 2004; Kraynak et al, 2018). These findings emphasize the critical need to better understand the mechanisms of sickness behavior in order to better treat both psychiatric and peripheral pathologies.

1.2 Interleukin-1β is a Key Initiator of Inflammation and Behavioral Changes

From the moment of discovery, IL-1 has revolutionized our understanding of innate immunity. Two ligands, IL-1α and IL-1β, collectively referred to as IL-1 were identified as a result of decades of research into endogenous pyrogens, that is the search for an endogenous factor capable of producing fever in the absence of infection (Dinarello, 2015). The existence of endogenous pyrogens was first proposed in 1943 by Eli Menkin who demonstrated that fever could be induced by injecting the supernatant of neutrophils isolated from sterile injury marking a major milestone in our understanding of fever and the host immune response. Menkin's findings were validated and expanded upon by his contemporaries however, over the next half century significant doubt remained within the scientific community as to whether it was the newly identified factor endogenous pyrogen or Lipid A contamination, the inflammatory properties of which were also being extensively investigated at the time, that was responsible for fever induction (Atkins, 1960). In parallel with research into endogenous pyrogen, investigations into cell derived factors which modulate biological function resulted in independent characterization of IL-1 under multiple names reflective of identified immunological roles such that by the 1970s, IL-1 was simultaneously being investigated as multiple distinct substances, each considered highly potent in their own regards, including: mononuclear cell factor (MCF), lymphocyte-activating factor (LAF), and B cell activating factor (BAF) among others (Oppenheim & Gery, 1993). The T cell activating quality of IL-1 was being investigated under the name lymphocyte activating factor (LAF) while the pyrogenic quality was being investigated as endogenous pyrogen or leukocyte pyrogen (Gery et al, 1972; Gery & Waksman, 1972). In 1979, through careful purification, the two compounds were found to be a single factor and renamed Interleukin-1 to reflect the multiple biological roles and

cellular targets of IL-1 (Rosenwasser et al, 1979). Lingering confusion over two apparently distinct forms of IL-1 with virtually identical signaling capacity were resolved in 1985 when the sequences of both IL-1α and IL-1β genes were described in humans (March et al, 1985).

IL-1β is produced as a 31 kDa, 269 amino acid, biologically inactive pro-protein which must undergo enzymatic cleavage to produce a biologically active 17 kDa form for secretion (Krumm et al, 2014). IL-1α by contrast, is produced in as a biologically active molecule which can be proteolytically cleaved in a similar manner to IL-1β to enhance signaling however it is not required. Because IL-1α is constitutively produced by barrier tissues and is typically released by cellular damage, it is often viewed as an alarmin (Garlanda et al, 2013). The most well defined mechanism by which IL-1β is proteolytically processed and released from the cell is through inflammasome mediated pyroptosis, a form of programmed cell death triggered by damage- or pathogen-associated molecular patterns (DAMPs and PAMPs respectively). In general terms, the inflammasome is a cytosolic multi-protein complex with enzymatic capabilities, formed by the activation of pattern recognition receptors (PRRs) which detect PAMPs and/or DAMPs. Once the inflammasome is assembled and enzymatically active, it is able to proteolytically process IL-1 family cytokines pro-IL-1β and pro-IL-18 to their biologically active forms as well as pro-Gasdermin D, the cleavage of which allows Gasdermin D pores to form on the plasma membrane, releasing active IL-1β, IL-18 and other cellular contents (Atianand et al, 2013; Ding & Shao, 2017). As pores form in the cell membrane and the cytosolic contents of the cell are emptied, pro-IL-1β may also be cleaved to its active form in the extracellular space via enzymes secreted by neutrophils, mast cells, and NK cells (Black et al, 1988; Coeshott et al, 1999; Hazuda et al, 1990; Li et al, 1995). Multiple distinct forms of inflammasome signaling, utilizing different PRRs and have been identified in a rapidly

developing area of study while alternative mechanisms for release of IL-1β without the need for cell death remain unclear. Nevertheless, inflammasome mediated pyroptosis is a critical means of IL-1β secretion, relevant in disease, injury, and even homeostatic contexts (Zengeler & Lukens, 2021). Although pyroptosis was initially thought to be exclusive to damage or pathogenic threat, recent reporting demonstrates that AIM2 inflammasome activation is critical during healthy brain development by mediating controlled neuronal cell death, the absence of AIM2, resulting in aberrant neuronal organization and increased anxiety-associated behaviors (Lammert et al, 2020). Additional lines of evidence including, PRR patterning in the brain throughout development and distinct behavioral phenotypes identified in multiple PRR knockout mice over the last decade indicate an emerging developmental role for pyroptosis and IL-1β signaling in the brain with significant behavioral consequences (Hung et al, 2018; Kaul et al, 2012; Okun et al, 2012; Okun et al, 2010).

Beginning in 1984 with Krueger's observation that IL-1β increases short wave sleep, behaviors associated with sickness expanded rapidly such that by the end of the 20th century, "sickness behavior" was firmly established as a set of pro-survival behaviors including: decreased locomotor activity, decreased social, sexual, and exploratory behaviors, anhedonia, decreased food and water intake, decreased grooming, and impairments in learning and memory (Dantzer, 2001). General proinflammatory insults such as LPS or viral mimetics are capable of inducing most or all behaviors listed as well as activate the stress-induced hypothalamic-pituitary-adrenal (HPA) axis, driving the production of proinflammatory cytokines IL-1β, IL-6, and TNFα in both blood and neural tissue as well as inducing a hormonal response driven by hypothalamic expression and secretion of corticotropin-releasing factor (Abraham & Johnson, 2009; Martin et al, 2013; McLinden et al, 2012). The HPA axis has long been studied

as a major driver of stress responses and is thought to underlie many sickness behavior phenotypes (Dantzer, 2018; Godoy et al, 2018; Hosoi et al, 2000). IL-1β administration alone is capable of eliciting all of these effects making it a valuable tool to study sickness behavior in its most simple form because despite its wide ranging signaling capacity, IL-1β possesses only a single signaling receptor facilitating precise experimental control of IL-1β signaling (Braun et al, 2012; Konsman et al, 2000; Matsuwaki et al, 2014). With a variety of robust models and a consistent phenotype, the first major questions for the field of sickness behavior research were, determining how cytokines enter the brain and mapping the causative neural circuits. Exhaustive efforts during the 1990s were able to identify brain regions activated by sickness however, the signaling pathways that mediate immune system influence over behavior remain mysterious (Wan et al, 1994).

IL-1β is produced naturally in the brain in response to peripheral inflammation, where—along with other inflammatory cytokines and paracrine signals—it is thought to influence behavioral shifts (Arnone et al, 2018; Cremona et al, 1998; Godbout et al, 2005; Konsman et al, 1999). Recent work has indicated that microglia, as the brain's resident immune cells, play a key role in sickness behavior through the secretion of paracrine signals (Dantzer, 2018; Zhu et al, 2019). Early investigations into the neuromodulatory capabilities of IL-1β utilized electrophysiology on neurons in slice culture and uncovered some of the most well studied roles for IL-1 in the brain although direct confirmation of mechanisms remains elusive. The ability of IL-1β to suppress long term potentiation (LTP) in hippocampal neurons has been reported since 1990 (Bellinger et al, 1993; Katsuki et al, 1990), the neurotoxic effects of IL-1β by NMDA induced excitotoxicity have also been reported since the early 1990s, and elucidation

of these mechanisms remains an area of active study today (Hewett et al, 1994; Taraschenko et al, 2021; Walker et al, 2019).

Modulation of the kynurenine metabolic pathway has been suggested as an indirect mechanism by which peripheral inflammation could influence neuronal activity without the necessity of cytokine signaling directly on neurons. In this model, IL-1β drives behavioral changes by inducing expression of Indoleamine-pyrrole 2,3-dioxygenase (IDO) which catalyzes the conversion of tryptophan to kynurenine, the first and rate-limiting step of the kynurenine pathway (O'Connor et al, 2009). Kynurenine can be transported to the brain by the large neutral amino acid transporter (LAT-1) where it is thought to contribute to a proinflammatory, neurotoxic environment via further metabolism by microglia and astrocytes (Walker et al, 2019).

The kynurenine pathway driven model of sickness behavior relies upon two primary lines of evidence. First, blockade of kynurenine transport into the brain has been shown to ameliorate LPS induced sickness behavior (Walker et al, 2019) and second, in response to peripheral LPS challenge, microglia upregulate IDO as well as downstream kynurenine pathway enzymes (Gonzalez-Pena et al, 2016) which convert kynurenine to 3-hydroxykynurenine and ultimately, quinolinic acid, an NMDA receptor agonist (Guillemin et al, 2007). The final two metabolites are also capable of generating reactive oxygen species which has been used as a rationale for studying the role of the kynurenine pathway in schizophrenia and depression (Dantzer et al, 2011). The role of the kynurenine pathway in sickness behavior and mood disorders remains a highly active and promising area of research however meta-analysis of depression studies has yet to uncover a link between kynurenine

levels and disease although the currently available data remains limited (Arnone et al, 2018; Metcalfe et al, 2018).

1.3 Routes of Inflammatory Signal Transduction to the Brain

The brain has been long viewed as an 'immune-privileged organ', a status shared with the eyes and testes, because unlike other tissues, the brain does not contain lymphatic vessels and the brain exhibits unique tolerance for allograft tissue which is quickly rejected in other organs (Medawar, 1948; Streilein, 2003; Zhao et al, 2014). In addition to a lack of tissue-penetrating lymphatic vessels, the central nervous system possesses a unique structure called the blood-brain-barrier (BBB). This structure is comprised of multiple cell types including and surrounding blood vessels, the purpose of which is to maintain ionic homeostasis and regulate access of blood-borne molecules to the brain (Stamatovic et al, 2008). As a result, under healthy conditions, molecules without specific active transport to the brain are generally unable to access the tissue. While some specific stimuli are known to disrupt or increase BBB permeability which could allow influx of cytokines to the brain, direct infusion of IL-1β does not disrupt BBB permeability (Banks & Kastin, 1992). Work from the lab of Abba Kastin in the early 1990s utilizing tagged and radiolabeled cytokines suggested that cytokines including IL-1α, IL-1β, IL-1ra (receptor antagonist), IL-6, and TNFα all may cross the BBB through saturable mechanisms i.e. active transport (Banks et al, 1994; Banks et al, 1991; Gutierrez et al, 1993; 1994). However, proteins responsible for active transport of these cytokines across the BBB have not been identified and these findings have yet to be verified using modern techniques, casting doubt over whether cytokines from the blood can truly access the brain. Once cytokines such as IL-1β and TNFα bind their receptors, the receptor can be internalized for degradation as a negative feedback mechanism, reducing circulating cytokines (Blanco et al, 2008; D'Alessio et al, 2005; Pan et al, 2007; Veluthakal et al, 2005). Therefore, it is possible that what was measured in early cytokine radiolabeling experiments was the internalized

cytokines present in endothelial cells. This notion is supported by their own findings that knockout of the TNF receptor eliminates entry of labeled TNFα to the brain however this does not preclude the possibility that transcytosis still occurs through unknown means (Pan & Kastin, 2002). Uncertainty surrounding the ability of IL-1β to cross the BBB led to several hypothesized routes of signal transduction which can be generally divided into two classes: neural and humoral.

The works of Robert Dantzer and Keith Kelley in 1995-1996 provided substantial evidence for a vagal route of IL-1β signal transduction to the brain (Bluthé et al, 1996b; Layé et al, 1995). Through a series of vagotomy experiments, they demonstrated that the Vagus nerve is one available route for IL-1β in the periphery to signal to the brain. Subdiaphragmatic vagotomy ablates the locomotor impairment associated with sickness behavior in response to intraperitoneal LPS or IL-1β injection however it has no impact on the behavioral effects of IL-1β when administered centrally or intravenously (Bluthé et al, 1996a; Layé et al, 1995). Subdiaphragmic vagotomy fails to prevent some symptoms such as anhedonia and weight loss induced by IL-1β, indicating multiple routes and/or multiple signals are needed to produce all aspects of sickness behavior (Wieczorek et al, 2005). Furthermore, Vagus nerve afferents are known to express IL-1R1 and respond to cytokine or even electrical stimulation which is sufficient to drive IL-1β mRNA expression in the brain, indicating a positive feedback mechanism and a brain specific signaling role for IL-1β in sickness behavior (Goehler et al, 1998; Hosoi et al, 2000; Konsman et al, 2000; Layé et al, 1995).

In the event of tissue damage or infection, IL-1β and other proinflammatory cytokines are released at the site of injury or pathological insult and enter circulation. Cytokines can stimulate the Vagus nerve which innervates much of the viscera and transmits an electrical

signal to the solitary tract nucleus (NTS) of the brain. Projections from the solitary tract then activate other brain regions including the central amygdala (CEA), ventrolateral medulla (VLM), parabrachial nucleus (PB), paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), ventromedial preoptic nucleus (VMPO), and the bed nucleus of the stria terminalis (BST) (Wan et al, 1994). Thus, in the neural route of signal transduction, IL-1β possesses at least two independent roles. It acts first in the periphery to stimulate the Vagus nerve, sending a cue to the brain that there is peripheral inflammation, inducing local production of IL-1β within the brain (Layé et al, 1995). Then the IL-1β produced in the brain is thought to act on neurons and glia by an IL-1R1 dependent mechanism to induce behavioral changes (Cremona et al, 1998; Deyerle et al, 1992). Despite the common conclusion that IL-1β must be activating neurons via the receptor IL-1R1, no direct evidence exists to support that, and it is unclear how IL-1R1 signaling would result in neuronal activation through canonical pathways. The discovery of a brain-restricted isoform of IL-1 receptor accessory protein with neuronal signaling capacity in vitro has fueled further speculation that IL-1β may have direct neuromodulatory capacity although direct evidence of this in vivo remains elusive (Davis et al, 2015; Gosselin et al, 2013; Huang et al, 2011; Smith et al, 2009). The insufficiency of vagotomy to ablate sickness behavior induced by i.v. administration of IL-1β indicates alternate routes exist for circulating IL-1β to act on the brain, collectively referred to as the "humoral route".

The humoral route of IL-1β signaling to the brain consists of three documented mechanisms with varying levels of support. First, IL-1β may cross the endothelium at sites of "leaky" blood brain barrier (BBB) such as the choroid plexus and circumventricular organs (CVOs) which include the median eminence, the organum vasculosum of the laminal terminalis

of the hypothalamus (OVLT), the area postrema in the brainstem, and the suprafornical organ (SFO) (Dantzer, 2001; Konsman et al, 2000). At these sites, IL-1β is capable of stimulating perivascular macrophages which then secrete IL-1β into the brain parenchyma (Carmichael et al, 2010). The second route of entry is mediated by endothelial cells within the brain which, bulk RNAseq data indicates are the highest expressers of IL-1R1 in the brain by an order of magnitude (Zhang et al, 2014b). IL-1β can act directly on the brain endothelium which stimulates secretion of IL-1β and other factors into the brain parenchyma by endothelial cells. Conditional knockout of IL-1R1 signaling (via MyD88) on endothelial cells is capable of ameliorating sickness behavior in some contexts and bone marrow transplant experiments emphasize the importance of this route while casting doubt on role of perivascular macrophages in sickness behavior (Ching et al, 2007; Knoll et al, 2017; Matsuwaki et al, 2014). Lastly, a third potential humoral route exists that proposes IL-1β can be actively transported across the BBB however only limited, indirect evidence supports this hypothesis. Radiolabeling experiments suggest that IL-1β can be transported into the brain via a saturable mechanism, indicating an active process however, it is unclear from this experiment whether the labeled IL-1β actually enters the brain parenchyma and there are no known transporters for IL-1β casting significant doubt on the existence of this mechanism (Banks et al, 1991). Therefore, the totality of the evidence suggests endothelial cells are the primary cellular target of IL-1β in the humoral route of signal transduction although multiple routes of signal transduction likely contribute to the induction of sickness behavior.

1.4 Lymphatic Vessels in the Meninges Facilitate Brain Solute Perfusion and Clearance

Lymphatic vessels embedded in the dura layer of the meninges were first described in humans in 1787 by Italian anatomist Paolo Mascagni however this structure largely evaded research interest until their 're-discovery' in 2015 (Aspelund et al, 2015; Bucchieri et al, 2015; Louveau et al, 2015; Mascagni, 1787). Highlighting a potentially distinct role from lymphatic vessels in other tissues, meningeal lymphatic vessels possess properties which distinguish them from other lymphatic vessels including a unique transcriptional signature and they do not undergo expansion in response to inflammation (Louveau et al, 2018). Although the route of lymphatic drainage had been unknown at the time, it had been reported that antigens delivered to the cerebrospinal fluid (CSF) could access the blood and stimulate an immune response in peripheral organs (Cserr et al, 1992; Harling-Berg et al, 1989; Knopf et al, 1995). Through extensive investigations by multiple groups, we now know that, CSF flows along perivenous spaces into the subarachnoid space of the meninges where it drains to lymphatic vessels which run alongside the dorsal superior sagittal, and transverse sinuses, and the dorsal sigmoid, and petrosquamosal sinuses, ultimately flowing to the deep cervical lymph nodes (Lee et al, 2020; Louveau et al, 2017). Since its rediscovery, meningeal lymphatic vessels have been described in rats and fish as well non-human primates and humans, indicating both high adaptive value and medical relevance (Absinta et al, 2017; Bower et al, 2017; Jung et al, 2017).

Description of the 'glymphatic' system, by which CSF circulating along paravascular routes approximates lymphatic drainage in the brain by collecting soluble debris, predates the rediscovery of meningeal lymphatic vessels however, these two concepts have since become closely intertwined as meningeal lymphatic vessels ultimately collect soluble tracer injected

into the CSF and drain to the deep cervical lymph nodes for immune surveillance (**Fig. 1**) (Iliff et al, 2012; Louveau et al, 2015; Ma et al, 2017). Glymphatic solute clearance is a dynamic process which is most active during sleep or anesthesia, and which different forms of sedation may differentially impact glymphatic function (Holth et al, 2019; Ozturk et al, 2021). Furthermore, meningeal lymphatic drainage of solute to the lymph nodes declines with age which may contribute to age-associated pathologies and effects on cognition (Ma et al, 2017).

In recent years, meningeal lymphatic vessels have been confirmed to be play a role in immune surveillance of the brain in both positive and negative immunological contexts. Ablation of meningeal lymphatic vessels in experimental autoimmune encephalitis (a murine model for multiple sclerosis) is able to reduce the T cell response and as a result reduce disease pathology (Louveau et al, 2018). By contrast, increased immune surveillance of the brain through experimental enhancement of meningeal lymphatic vessels augments the T cell response to glioblastoma, boosting the efficacy of checkpoint blockade therapy and enabling tumor clearance by the immune system (Hu et al, 2020; Song et al, 2020).

In addition to providing the conventional role of lymphatic vessels in facilitating immune surveillance by the adaptive immune system, meningeal lymphatic vessels contribute to glymphatic clearance of solute within the CSF and interstitial fluid (ISF), most notably amyloid-β which can form protein aggregates in the brain that are a hallmark of Alzheimer's Disease pathology, indicating there may be important cognitive and behavioral implications to meningeal lymphatic function (Da Mesquita et al, 2018b; Iliff et al, 2012). Our group has previously demonstrated that meningeal lymphatic ablation can exacerbate the accumulation of amyloid-β aggregates in the brain of transgenic Alzheimer's Disease mice, and impair cognitive function of wildtype adult mice as assessed by the Morris water maze, a learning and memory

test, while experimental enhancement of meningeal lymphatic vessels is able to improve aged mouse cognition as assessed by the same test (Da Mesquita et al, 2018b).

The involvement of meningeal lymphatic deterioration and dysfunction in aging is of particularly keen research interest based on reporting that meningeal lymphatic drainage declines with age while enhancement can ameliorate cognitive deficits in aged mice, as well as additional lines of evidence which suggest that age-associated lymphatic/glymphatic decline in humans contributes to multiple age-related pathologies (Da Mesquita et al, 2018b; Ma et al, 2017; Paradise et al, 2021). Reports of glymphatic decline with age and a connection to protein aggregate pathologies in animal models predate the rediscovery of meningeal lymphatic vessels however, recent reports generated from clinical patient data confirm that hallmarks of glymphatic decline are present in aging patients and significantly correlate with cognitive impairment (Kress et al, 2014; Ohashi et al, 2021; Paradise et al, 2021). The relationship between sleep, glymphatic function, and aging is the subject of ongoing speculation and research as sleep disruption is a common comorbidity found in age-associated neurodegenerative disorders (Malhotra, 2018). Due to the established effect of sleep on glymphatic activity, disruptions in sleep may be a compounding factor, along with meningeal lymphatic decline, in overall glymphatic dysfunction and subsequent neurodegeneration (Nedergaard & Goldman, 2020). Additionally, problems with sleep are associated with increased incidence of psychiatric illness, cognitive impairment, and neurodegeneration throughout life indicating a potential glymphatic component in these pathologies (Leigh et al, 2015; Mander et al, 2013; Spira et al, 2014).

While there are numerous compelling disease contexts wherein the role of meningeal lymphatic function remains to be explored, a notable gap remains in our understanding of

sickness behavior and how it may tie into psychiatric disease and cognition. Sickness behavior is suspected to rely on the perfusion of soluble paracrine signals including cytokines, prostaglandins, and glucocorticoids which logically should be subjected to the same glymphatic forces which mediate clearance of amyloid-β (Crestani et al, 1991; Matsuwaki et al, 2014). Therefore, disruption of meningeal lymphatic vessels may impair critical signaling events through multiple means. By impairing meningeal lymphatic function, paracrine signals may either be delayed or prevented from reaching their target cell populations, resulting in insufficient activation of a target cell population. Conversely, paracrine signals may fail to be efficiently cleared from the interstitial spaces resulting in prolonged or hyperactivation of target cell populations. These scenarios are not mutually exclusive therefore, a third possibility exists in which lymphatic disruption results in simultaneous over stimulation and under stimulation of discrete compartments of the brain.

1.5 Connections Between Sickness Behavior and Psychiatric Disease

The mechanisms by which the immune system informs the brain of threats to modulate behavior is a rapidly developing field with that may be intimately linked with a variety of psychiatric disorders. As one of the first observed IL-1β influenced behaviors, the role of IL-1β in promoting short wave sleep has been extensively investigated. Included within the discussion of Krueger's 1984 report on the short wave sleep enhancing effects of IL-1β, he considers the possibility that IL-1β may be involved in the regulation of homeostatic sleep but also acknowledges a clear contradiction that must be reconciled (Krueger et al, 1984). A body temperature decrease is associated with homeostatic sleep while IL-1β is known to induce fever. Despite this potential paradox, new compelling lines of evidence continued to arise suggesting a role for IL-1β in homeostatic sleep. IL-1β concentration in both serum and CSF has been shown to follow a circadian rhythm, increasing during the sleep cycle in humans, rodents and cats (Cearley et al, 2003; Lue et al, 1988; Moldofsky et al, 1986). Additionally, IL-1β mRNA expression increases in the brain parenchyma of rats during sleep deprivation (Mackiewicz et al, 1996). Krueger did not discount the notion that IL-1β expression may be highly localized and regulated differently under homeostatic conditions and recent evidence suggests that may be the case. The use of microinjections into specific brain nuclei has revealed highly location dependent effects of IL-1β. Multiple lines of evidence exist to show IL-1β influences firing rates of neurons in several sleep related nuclei including the Dorsal Raphe Nucleus, Laterodorsal Tegmental Nucleus (LDT), and Preoptic Area of the hypothalamus (Alam et al, 2004; Brambilla et al, 2010; Brambilla et al, 2007; Tabarean et al, 2006). Microinjection of IL-1β is capable of enhancing non-REM sleep in all three nuclei however, supporting the hypothesis that IL-1β may have diverse location dependent functions, different neuronal effects

have been observed in each implicated nucleus. In the LDT, the sleep promoting effects of IL-1β have been attributed to inhibition of cholinergic neurons (Brambilla et al, 2010). IL-1β inhibition of serotonergic neurons has been implicated in the DRN and the putative neuronal population that responds to IL-1β in the preoptic area has not yet been identified (Brambilla et al, 2007). These studies primarily rely on electrophysiological recordings in slice culture and behavioral analysis following microinjection to make their claims which does not preclude the possibility that IL-1β acts indirectly through a glial mediator however, regardless of mechanism, IL-1β has a well-documented neuromodulatory capacity.

Post-traumatic stress disorder (PTSD) was one of the earliest psychiatric disorders to be associated with abnormal concentrations of circulating IL-1β when in 1997, and Israeli group documented elevated levels IL-1β in serum correlates with duration of disease (Spivak et al, 1997). Abnormalities of the immune system are now well documented in PTSD patients. Peripheral blood mononuclear cells (PBMCs) of PTSD patients will spontaneously produce more pro-inflammatory cytokines, including IL-1β compared to healthy controls (Gola et al, 2013). This increase in IL-1β production may extend to the CNS as chronic stress has been shown to alter microglia morphology and induce an inflammatory state (Tynan et al, 2010). Similar aberrations in pro-inflammatory cytokine production can be detected in the CSF of PTSD patients responding to painful stimuli (Lerman et al, 2016). Nearly two decades later, this phenomenon was used as a basis for studying the role of IL-1β in stress enhanced fear learning (SEFL). Remarkably, central inhibition of IL-1β signaling by infusion of IL-Ra completely eliminates SEFL although the mechanism remains unknown (Jones et al, 2015).

Aberrant immunological profiles have been identified in mood disorders patients including with Major Depressive Disorder since the 1990s however, reflecting the highly complex nature

of the disease, many early and contemporary reports offer conflicting results (Weizman et al, 1994). Genome wide association studies have identified polymorphisms in the IL-1β gene associated with increased risk of developing childhood depression, increased risk of developing geriatric depression, and reduced response to anti-depressant therapy (Baune et al, 2010; Hwang et al, 2009; Ridout et al, 2014). These findings remain controversial as other studies have failed to identify genetic polymorphism of IL-1β associated with childhood depression however it is possible that IL-1β polymorphisms merely predispose individuals to be more susceptible to develop depression in the presence of other contributing factors which may be inconsistent across different investigations (Misener et al, 2009; Misener et al, 2008). Nevertheless, circulating IL-1β has been identified as a biomarker of depression generating interest in IL-1β-targeted therapeutic strategies for depression (Ellul et al, 2016; Lu et al, 2013; Martinez et al, 2018; Mota et al, 2013).

The involvement of IL-1β in addiction and substance abuse related pathologies is highly complex due to the multiple overlapping domains of IL-1β activity in any given patient. The high rate of comorbidity between substance abuse and mood disorders is one complicating factor as mood disorders have also been associated with IL-1β dysregulation (Turner et al, 2018). Additionally, the route of administration for the substance of abuse and risky behaviors associated with substance abuse have the potential to induce inflammation and IL-1β production that could obscure a causal role. Nevertheless, a growing body of evidence describes an important neural role for IL-1β signaling in driving substance seeking behaviors.

The link between IL-1β and addiction in the context of alcoholism was first uncovered by genetic association studies which found that polymorphisms in the IL-1 family ligand genes IL-1α and the antagonist, IL-1Ra, were associated with alcoholism (Saiz et al, 2009). Analysis

of human post-mortem brain tissue found that IL-1R1, the sole receptor for IL-1β, is overexpressed in alcoholics along with other markers related to stress and inflammation (McClintick et al, 2013). Attempts to model the involvement of IL-1 in alcohol seeking behavior have largely been successful as alcohol seeking behavior is attenuated in IL-1R1 knockout mice and direct administration of IL-1Ra into the central amygdala dramatically reduces voluntary alcohol consumption while antagonist administration to neighboring nuclei has no effect (Karlsson et al, 2017; Marshall et al, 2016). Similar observations have been found in the context of cocaine abuse in both humans and mice. Circulating IL-1β positively correlates with severity of cocaine dependence in patients (Araos et al, 2015). In mice, chronic cocaine use drives IL-1β mRNA expression in the ventral tegmental area of the brain and IL-1Ra mediated inhibition of IL-1 signaling in the same region can decrease drug-seeking behavior in a dose dependent manner (Brown et al, 2018).

Substance abuse and opioid addiction in particular are associated with centrally mediated immunosuppression, characterized by a suppression of iNOS production in response to inflammatory stimuli (Reece, 2008; Szczytkowski et al, 2013). Opioid induced immunosuppression can be linked to unrelated stimuli by Pavlovian conditioning (Hutson et al, 2017). Based on the established link between Pavlovian conditioning and IL-1 signaling in the brain, the lab of Donald T. Lysle at the University of North Carolina at Chapel Hill is extensively investigating the role of IL-1β in models of heroin-conditioned immunosuppression and found IL-1β signaling in the dorsal hippocampus is necessary to induce the phenomenon (Lebonville et al, 2016; Szczytkowski et al, 2013). Additional IL-1β signaling is required in the basolateral amygdala to produce heroin-conditioned immunosuppression, indicating IL-1β

may link multiple addiction related pathologies (Hutson et al, 2017). Whether this role is distinct from classical Pavlovian conditioning remains an open question.

Our understanding of the relationship between immune system perturbations and Autism Spectrum Disorder (ASD) is rapidly evolving. The association between immune perturbations and ASD was first identified based on observations that children who contracted Rubella during gestation were at a dramatically higher likelihood to display ASD symptoms, and further studies confirmed that maternal infection in general is associated with increased risk of ASD (Atladóttir et al, 2010; Chess, 1977). As a result, maternal immune activation (MIA) is an established risk factor for ASD and the basis for an animal model of psychiatric disease including ASD, in which the timing and severity of MIA, produces distinct social and behavioral abnormalities in mice (Choi et al, 2016; Meyer et al, 2006). Although the developmental aspects of ASD may be beyond the scope of sickness behavior, behavioral symptoms may be due in part to an aberrant response to immunological signals. ASD patients and MIA mouse models have a distinctly altered immunological profile characterized by elevated circulating IL-17 and increased Th17 cells (Eftekharian et al, 2018; Gupta et al, 1998; Hsiao et al, 2012). Additionally, fever is associated with temporary alleviation of ASD behavioral symptoms, establishing a direct link between immune system status and ASD behavioral pathology (Curran et al, 2007). A variety of cytokines are now known to impact behavior though direct mechanisms remain largely unknown. Reporting from our group has demonstrated a critical role for interferon gamma (IFNγ) in mouse social behavior, the absence of which diminishes social preference and alters neuronal connectivity (Filiano et al, 2016). Similarly, the loss of IL-15 signaling has been reported to alter sociability, anxiety-like behavior, and serotonin signaling in the brain among additional broad metabolic phenotypes

(He et al, 2010; Wu et al, 2010; Wu et al, 2011). Further anxiety-associated abnormalities have been reported in IL-33 knockout mice, and recent reporting from our group has demonstrated that IL-17a secreted by meningeal γδ T cells influences exploratory behavior through direct action on neurons (Alves de Lima et al, 2020; Dohi et al, 2017). The precise nature of when and how cytokines influence behavior, and how these mechanisms are disrupted in ASD remain unclear and the target of continuing research.

1.6 Thesis Rationale

Sickness behavior is a virtually unavoidable aspect of the human condition to the extent which many characteristics of sickness behavior could be inferred by a layperson based on personal experience, without a technical definition. Transcending the human experience, sickness behavior can be readily identified in a variety of animal species by simple behavioral observations which is undoubtably the impetus for countless veterinarian appointments. Despite the ubiquitous nature of sickness behavior in society and a common understanding of its broad symptoms, we possess little technical understanding of how signals generated by the immune system in peripheral tissues ultimately modulates neuronal activity and thus behavioral output.

Mood disorders are highly prevalent in the United States, affecting more than one in five adults in their lifetime while current therapies are only modestly effective, and their underlying mechanisms remain mysterious (Kessler et al, 2005; Merikangas et al, 2010). In recent years, increasing numbers of publications have suggested an overlap between the diagnostic criteria for mood disorders and the set of symptoms common to any immunological threat, collectively referred to as "sickness behavior" (Dantzer, 2018; Martinez et al, 2018). These symptoms are conserved throughout vertebrate species and broadly include decreased motivation, lethargy, and impaired concentration. Additionally, clinical data has shown major depressive disorder patients have elevated levels of plasma IL-1β, a key initiator of sickness behavior (Koo & Duman, 2009; Mota et al, 2013). These findings have generated speculation that the immune system could become a therapeutic target for mood disorders although, it remains unclear how inflammatory signals originating from outside the brain ultimately modulate behavior at the neuronal level. Nevertheless, newly uncovered connections between IL-1β signaling, sickness

behavior and mood disorders underscore the urgency of understanding the mechanisms of this behavioral response as any novel insights into sickness behavior may facilitate new therapeutic strategies.

The broad expression of IL-1R1, the sole receptor for IL-1β, within multiple cell populations of the brain, has generated decades-long speculation that IL-1β signaling within the brain plays a role in sickness behavior and potentially other homeostatic functions (Deyerle et al, 1992; Rosenberg et al, 2018; Zhang et al, 2014a). This speculation is bolstered by electrophysiology data from slice culture, demonstrating the ability of IL-1β to inhibit long-term potentiation in hippocampal neurons which may contribute to learning and memory by influencing neuronal energy regulation however, conclusive evidence demonstrating that direct neuromodulatory capabilities of IL-1β are relevant in the context of sickness behavior remains elusive (del Rey et al, 2013; Del Rey et al, 2016; Katsuki et al, 1990).

The recently discovered meningeal lymphatic system represents a potential point of access to the brain from the immune system that remains unexplored in the context of sickness behavior (Louveau et al, 2015). Publications from our lab have established that meningeal lymphatic vessels facilitate solute clearance from the brain parenchyma and that these vessels deteriorate with age resulting in accumulation of protein aggregates as well as cognitive impairment (Da Mesquita et al, 2018b). Additionally, age is a significant factor governing the severity of sickness behavior and late-life mood disorder diagnoses are strongly correlated with worse outcomes (Godbout et al, 2005; Prina et al, 2013). These findings have spurred speculation that meningeal lymphatic function, sickness behavior, and psychiatric illness may be linked together therefore, a thorough understanding of meningeal lymphatic function and sickness behavior is essential to developing new strategies to treat cognitive decline and mood

disorders.

To summarize, the basis of this thesis is to address the following three experimental questions:

1. **What are the cellular targets and sources of IL-1β in the CNS involved in the behavioral response to peripheral inflammation?**

2. **Do meningeal lymphatic vessels contribute to the behavioral response to peripheral inflammation?**

3. **Does meningeal lymphatic impairment contribute to the exaggerated response to peripheral inflammation observed in aged mice?**

1.

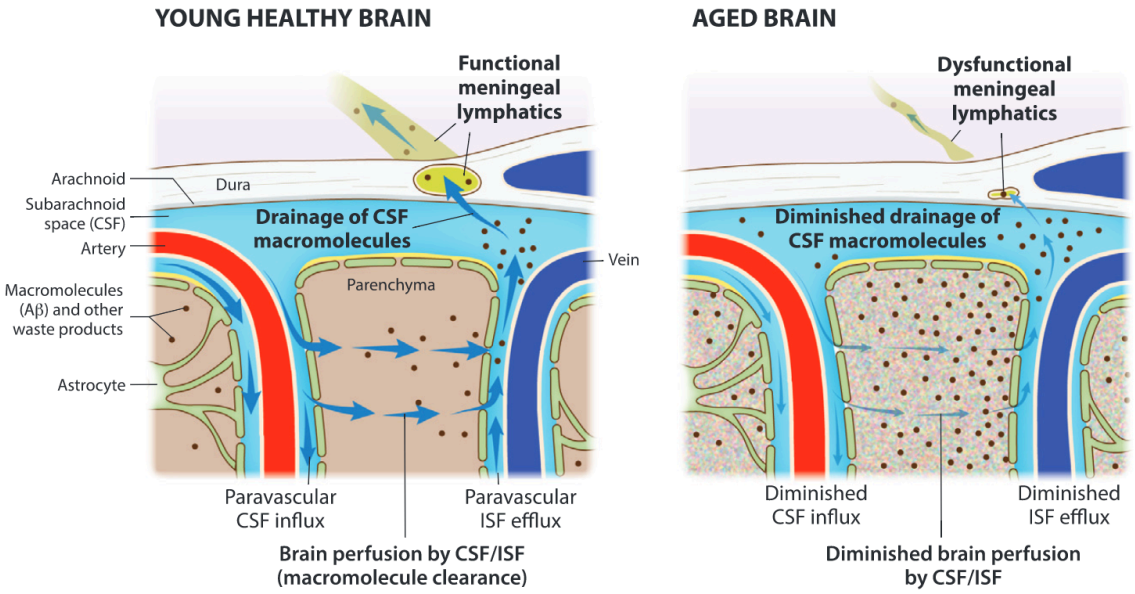

Figure 1. Schematic representation of lymphatic/glymphatic impairment associated with advanced age. Soluble macromolecules are cleared from the brain parenchyma by perfusion of CSF/ISF from arterial paravascular spaces to venous paravascular space where they are ultimately drained to meningeal lymphatic vessels. Age associated deterioration of meningeal lymphatic vessels impairs CSF/ISF perfusion and solute clearance from the brain.

Adapted from (Da Mesquita et al, 2018a)

Chapter 2 – Results

2.1 Interleukin-1β Signaling Within the Brain in Not Required to Modulate Behavior

To study sickness behavior, we developed a treatment and behavioral testing paradigm designed to simplify the biological response while retaining physiological relevance and capturing multiple facets of sickness behavior in mice. Intraperitoneal (i.p.) delivery of IL-1β was selected as a model of peripheral inflammation-induced sickness behavior for two key reasons. First, direct cytokine injection simplifies the biological response relative to live infection because IL-1β is detected by a single signaling receptor (IL1R1) while remaining capable of inducing robust sickness behavior (Dinarello, 1994). Second, we made the judgement that i.p. delivery of IL-1β is the most physiologically relevant route to study as it can be viewed as an experimental approximation of IL-1β released due to infection or injury in peripheral tissue. Reports have demonstrated that IL-1β introduced by a variety of routes, including direct infusion into the brain or cerebrospinal fluid, intravenous, and subcutaneous, all result in similar behavioral outputs but may rely on distinct signaling pathways (Bluthé et al, 1996a; b). Each of these routes of IL-1β administration may be relevant in specific disease contexts however, we believe that the intraperitoneal route simulates the broadest range of injuries which may induce sickness behavior.

To collect behavioral data, we subject mice to repeated 10-minute open field test at 1, 2, 3, and 6 hours following IL-1β or saline vehicle control i.p. injection (**Fig. 2a**). The open field test can be used to assess both activity and anxiety by measuring both total distance traveled and time spent in the center of the arena which mice typically explore but prefer the relative safety of high walls (Swiergiel & Dunn, 2007). This strategy enables us to capture locomotor activity over time so we can assess the severity and duration of IL-1β-induced behavioral changes. This model induces short-term depression of locomotor activity that generally

resolves by the 6th hour following IL-1β injection (**Fig. 2b**). Due to the established confounding nature of assessing anxiety during reduced locomotor activity, we did not consider time spent in the center of the arena as a meaningful measure of anxiety in IL-1β injected mice. Although distance traveled is the most definitive measure of overall activity, the possibility exists that mice with distinct activity phenotypes could appear the same by this measure. For example, a sluggish mouse that moves consistently but slowly could travel the same distance as a mouse that rests for a majority of the test but moves energetically for short periods. To address this possibility, we quantify time immobile for each mouse (**Fig. 2c**) however, we do not observe any divergence between trends detected by measuring activity versus immobility therefore, we will report distance traveled as our primary measurement of activity, as the most sensitive and scientifically established metric. One caveat to our experimental approach that should be acknowledged is that control mice become less active in subsequent tests with repeated exposure to the open field testing environment that reaches a base line at 6 hours and persists through the next day (**Fig. 2d, e**). We speculate that this effect is due to increased familiarity and therefore reduced exploratory interest. Sickness behavior has been described as a motivational shift and as such, we are measuring a general decrease in typical activity which includes exploratory interest (Dantzer, 2001).

To investigate the persistent notion that IL-1β signaling within the brain contributes to sickness behavior, we generated mice with conditional knockout of *Il1r1*, the gene that encodes the sole signaling receptor for IL-1β. To achieve conditional ablation of IL-1R1 from brain endothelial cells, we used AAV-BR1-Cre, which specifically targets brain endothelium (**Fig. 3a)**. Deletion of IL-1R1 from several brain-resident cell types was achieved by crossing $Il1r1^{flox/flox}$ mice with neuron-specific ($Syn1^{Cre}$, **Fig. 3b**), astrocyte-specific ($Gfap^{CreERT2}$, **Fig.**

3c) or microglia-specific (*Cx3cr1*^{CreERT2}, **Fig. 3d**) mice. For inducible Cre lines, deletion was induced by 2-week course on tamoxifen diet followed by 2 weeks on regular chow prior to behavioral testing. We found that none of these conditional knockout mice sufficed to alter sickness behavior in response to peripheral IL-1β or altered baseline activity.

We further assessed whether local IL-1β signaling within the brain parenchyma contributes to sickness behavior using *Il1b* knockout mice which retain the ability to respond to IL-1β while lacking the capacity to produce IL-1β endogenously. Therefore, we can experimentally control the amount of IL-1β, and where it originates from, in these mice. We confirm that i.p. injected IL-1β can access the bloodstream of *Il1b* knockout mice and circulate at levels equivalent to wildtype controls during the timeframe of sickness behavior (**Fig. 4a**). Furthermore, these mice exhibit a typical behavioral response to peripheral IL-1β indicating that IL-1β production within the CNS is not required to induce sickness behavior (**Fig. 4b**).

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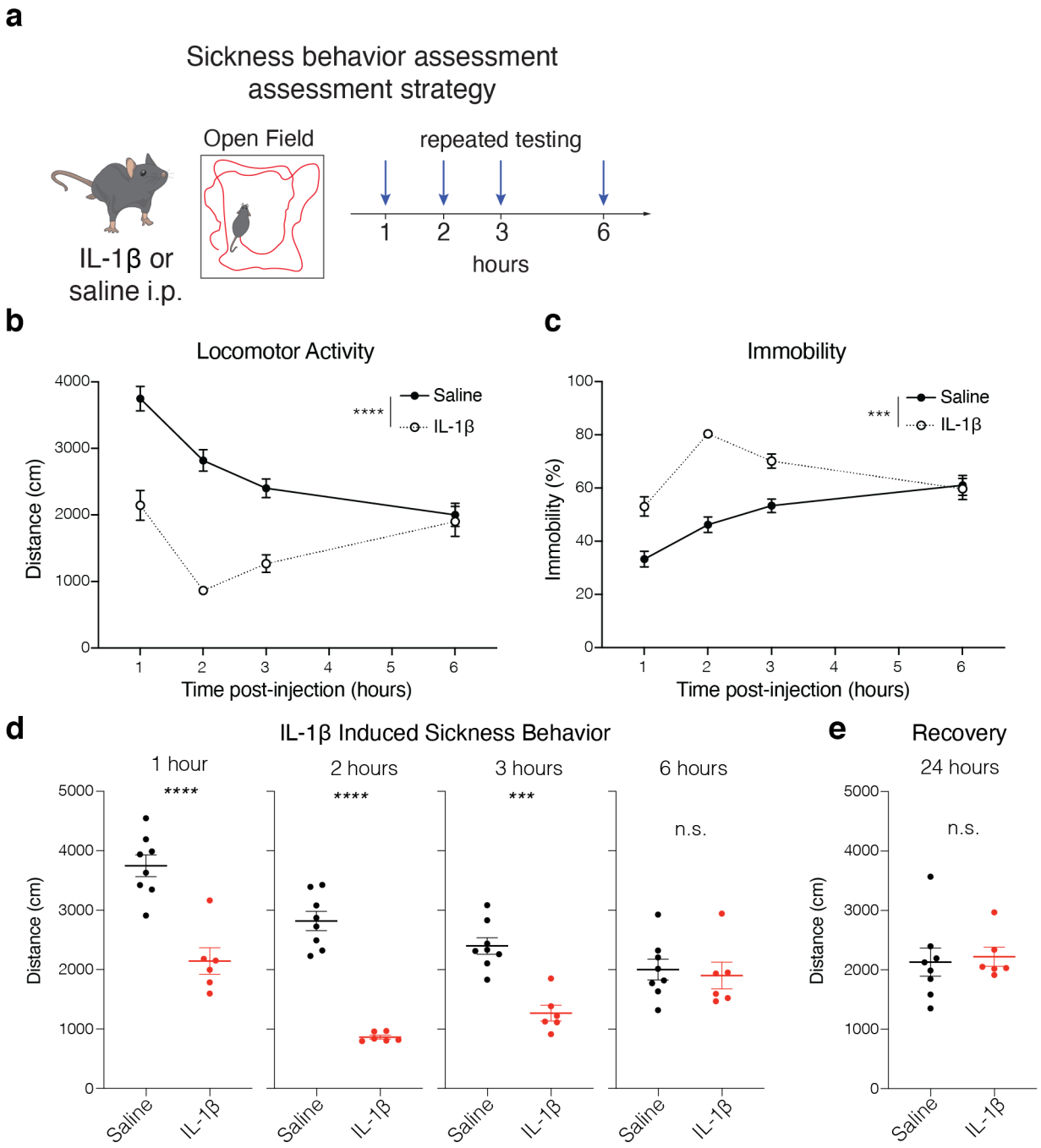

Figure 2. Experimental approach to measure sickness behavior. (**a**) Mice are treated with 1µg IL-1β or saline vehicle is delivered via i.p. injection then subjected to multiple 10-minute open field tests. Sickness behavior is assessed at 1, 2, 3, and 6 hours post injection, an additional test is performed 24 hours post injection to detect any prolonged effects. (**b**) Summary of total distance traveled at each timepoint. (**c**) Summary of time immobile at each time point. (**d**) Distance traveled at each time point assessing sickness behavior, and (e) recovery. Data represented as mean ± SEM; n=6-8, ***p<0.001 ****p<0.0001, 2-way ANOVA with repeated measures and Šidák correction for multiple comparisons.

3.

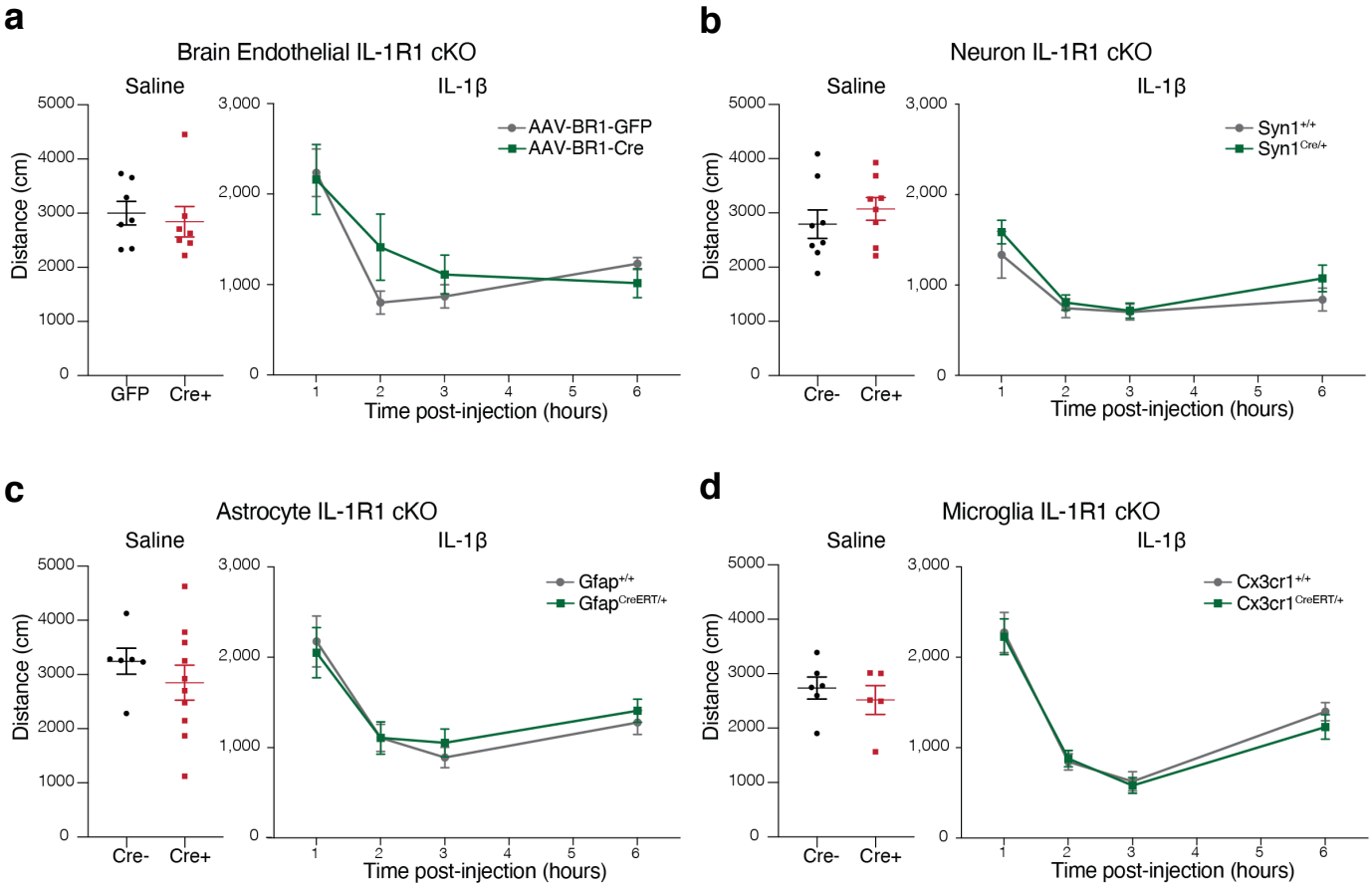

Figure 3. Conditional deletion of IL1R1 on neural cell populations does not impact peripheral cytokine induced sickness behavior. (**a-d**) Locomotor activity following saline (left) or IL-1β i.p. injection (right) in conditional knockout mice. (**a**) Brain endothelium was targeted by injection of AAV-BR1-GFP or AAV1-BR1-Cre into *IL1R1*$^{fl/fl}$ mice (n=7 per group), or (**b-d**) by crossing floxed mice to cell specific Cre lines: (**b**) neurons (*Syn*^{Cre}, n=8 mice per group), (**c**) astrocytes (*Gfap*^{CreERT2}, n=6-10 mice for saline, n=13-15 mice for IL-1β), (**d**) and microglia (*Cx3cr1*^{CreERT2}, n=5-6 mice for saline, n=11-17 mice for IL-1β). Data is presented as mean ± SEM; no significant difference was found between groups. Representative of two independent experiments.

4.

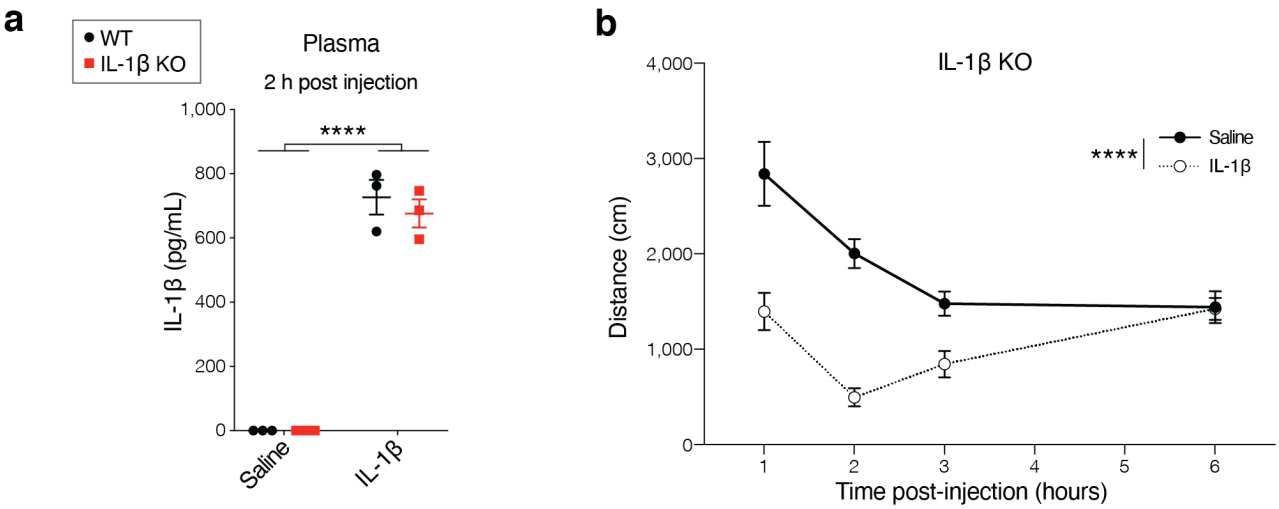

Figure 4. IL-1β production within the CNS is not required to induce behavioral shift in response to peripheral IL-1β. (**a**) IL-1β detected in plasma of IL1B KO mice and wildtype controls 2 hours following i.p. injection of 1μg IL-1β or saline. (**b**) Locomotor activity of IL1B KO mice following IL-1β or saline i.p. injection. Data is presented as mean ± SEM; (**a**) n=3, each point represents plasma pooled from 2 biological replicates, (**b**) n=8, ****p<0.0001 2-way ANOVA Representative of two independent experiments (**b**)

2.2 Experimental Manipulation of Meningeal Lymphatic Vessel Function Alters the Microglial and Behavioral Responses to Peripheral Inflammation

To address the possibility that meningeal lymphatic vessels play a role in sickness behavior, we ablated meningeal lymphatics in adult (2-month-old) mice using the photodynamic drug Visudyne (verteporfin) which, upon targeted photoactivation, selectively ablates meningeal lymphatic vessels (Da Mesquita et al, 2018b). Following intra cisterna magna (i.c.m.) injection of 5μL verteporfin solution, the drug accumulates in lymphatic vessels where it is photoactivated through intact skull at five locations previously described to effectively target lymphatic vessels (Da Mesquita et al, 2018b). For sham procedure, photoactivation is not performed. Following one-week recovery after lymphatic ablation or sham procedure, we assess sickness behavior by repeated open field testing following i.p. injection with IL-1β or saline (**Fig. 5a-c**). Lymphatic ablation was found to have no impact on anxiety, exploratory behavior or overall locomotor activity of mice injected with vehicle as a control (**Fig. 5c**), whereas lymphatics-ablated mice injected with IL-1β exhibited a marked reduction in locomotor activity (**Fig. 5d**). We verify that this effect is due to lymphatic ablation and not off-target effects of the Visudyne solution or photoactivation by assessing sickness behavior in Visudyne i.c.m. injected mice where photoactivation was purposefully performed off-target from known lymphatic vessels (**Fig. 6**). In this instance, artificial cerebrospinal fluid (aCSF) i.c.m. injection was used in lieu of Visudyne for sham treatment to assess any baseline impact of Visudyne solution injection on behavior however, no difference was detected between baseline activity or response to peripheral IL-1β, bolstering our assessment that the impact we observe on the behavioral response to IL-1β in our lymphatic ablation model is not due to off-target effects.

As the tissue resident immune cell of the CNS, microglia are known to respond to peripheral inflammation however, the relationship between microglia activity and behavioral output remains unclear (Wynne et al, 2010). We employ a dual analysis pipeline to interrogate the impact of meningeal lymphatics ablation on microglia response to peripheral inflammation, by harvesting brains from lymphatics-ablated or sham-treated mice 2 hours after injection of IL-1β or saline. Each brain was divided into hemispheres to enable concurrent histological and transcriptional profiling of microglia. Microglial morphology is dynamic and responsive to immune activation; under homeostatic conditions microglia are highly ramified, whereas upon activation, they become simplified and amoeboid (Kettenmann et al, 2011). Quantification of microglial complexity by Sholl analysis revealed that ablation of meningeal lymphatics impairs the morphological shift of microglia to an activated form. In addition, we also observed a less ramified morphology from vehicle-treated, lymphatics-ablated mice relative to sham controls, indicative of elevated inflammation at baseline (**Fig. 7a, b**).

To assess microglia activation on a transcriptional level, we employed magnetic-activated cell sorting (MACS) to isolate and sequence $CD11b^+$ cells from the other hemisphere of lymphatics-ablated and sham-operated mice 2 h following saline or IL-1β injection (**Fig. 8a**). This approach yielded ~84% microglia, ~11% macrophages, and ~5% endothelial cells as determined by expression of marker genes (**Fig. 8b**). MACS sorting was selected over alternative, more specific cell sorting techniques such as fluorescence-activated cell sorting (FACS) to ensure that data was generated from the highest quality samples. FACS sorting requires additional time and subjects cells to increased hydrodynamic stress which can degrade sample quality by inducing an unintended transcriptional response (Bohlen et al, 2019; Heng et al, 2021). We identified five microglial clusters, of which cluster 2 showed a high number of

upregulated genes associated with an activated state (**Fig. 8c, d**). Notably, cluster 2 was greatly expanded in IL-1β-treated groups but this expansion diminished with lymphatic ablation (**Fig. 9a, b**). Broadly speaking, the microglial transcriptional response to IL-1β under sham and meningeal lymphatics-ablation is similar, sharing 60% of the top 25 differentially expressed genes albeit with a lower log fold change in meningeal lymphatics ablation (**Table 1, 2, Supplementary file**). *Il1b*, *Tnf*, and *Ptgs2 (*prostaglandin-endoperoxide synthase 2, commonly known as COX-2), genes previously implicated in sickness behavior, were found elevated in microglia after induction of peripheral inflammation with IL-1β, but not *Il6* (**Fig. 9c**). Furthermore, we observe significant differences in overall gene expression due to meningeal lymphatic ablation in the absence of IL-1β stimulation, for example, elevated *Tnf* expression was associated with lymphatic ablation at baseline but no difference was detected between sham and ablation with IL-1β treatment (**Table 3**, **Fig. 9c**). It should be noted that significantly elevated *Il1b* expression in lymphatics-ablated mice in response to peripheral inflammation was the result of higher expression levels in fewer cells, as *Il1b* expression was detected in more than 32% of sham microglia responding to peripheral IL-1β, but in fewer than 25% of lymphatics-ablated microglia responding to IL-1β (**Supplemental file**). Consistent with their reduced overall shift to an activated state, we observe a diminished number of differentially upregulated and downregulated genes in response to peripheral IL-1β in the lymphatics-ablated mice, and the top 25 differentially expressed genes between sham response to IL-1β and meningeal lymphatics ablation response to IL-1β are universally decreased in meningeal lymphatic ablation (**Fig. 9d, Table 4**). To investigate how this differential transcriptional response may impact microglia function, we identified enriched biological processes in differentially expressed genes unique to either the sham or meningeal lymphatics ablation

response to peripheral IL-1β and following saline. It is important to note that a large number of enriched biological pathways (189 downregulated, 920 upregulated) were detected in meningeal lymphatics ablation microglia relative to sham in the absence of IL-1β stimulation, which are broadly indicative of elevated baseline inflammation and potentially indicate an impact on circadian rhythm (**Table 5, 6, Supplementary file**). Overall, we observe a stark decrease in the number of unique enriched pathways (302 in sham and 42 in ablation), the number of genes identified per pathway, and the relative statistical strength of enrichment (**Fig. 9e, Table 7, 8, 9, 10, Supplementary file**). The most statistically significant uniquely enriched pathways in sham microglial IL-1β response genes were involved in metabolic processes such as "ATP metabolic process", "Mitochondrion organization", "Mitochondrial respiratory chain complex assembly", "Generation of precursor metabolites and energy", and "Oxidative phosphorylation" indicative of elevated cellular activity. Conversely, uniquely upregulated biological processes in microglial IL-1β response genes after lymphatic ablation tend to concern the immune response, including: "Cytokine secretion", "Lymphocyte activation involved in immune response", "T cell differentiation involved in immune responses", "Cellular response to interferon-alpha" and "Myeloid leukocyte activation" (**Fig. 9e**, **Supplemental file**).

Due to our unexpected finding that more severe peripheral cytokine-induced sickness behavior is associated with impaired microglia response, we further investigated how near total depletion of microglia would impact sickness behavior. To examine the impact of peripheral inflammation on mouse behavior in the absence of microglia, we added the CSF1R antagonist PLX5622 into the feed for 2 weeks which has been previously established to deplete >99% of microglia as well as other tissue resident macrophages (**Fig. 10a, b**) (Dagher et al, 2015). When sickness behavior was assessed, we observed a heightened response to peripheral inflammation

in microglia-depleted mice, suggesting a potential role for microglia in limiting the severity of sickness behavior (**Fig. 10c**). Although this finding is contrary to the widely speculated role of microglia in sickness behavior, it has been independently confirmed with a peripheral LPS-induced sickness behavior model in a recent report (Dantzer, 2018; Vichaya et al, 2020).

Meningeal lymphatic function has been reported to decline with age which we speculate may contribute to age-associated exacerbation of sickness behavior (Da Mesquita et al, 2018b; Ma et al, 2017). To identify similarities between the effects of aging and of meningeal lymphatics ablation on the microglial response to peripheral inflammation, we sequenced $CD11b^+$ cells from the brains of aged (2-year-old) mice 2 hours after injection of IL-1β or saline and integrated the datasets with the previous adult (2-month-old) mouse meningeal lymphatic ablation dataset (**Fig. 11a, b, Fig. 12a**). A comparison of the IL-1β response-associated transcriptional changes across three conditions—young (sham), young with lymphatic ablation (ablation), and geriatric (aged)—revealed fewer upregulated genes and more downregulated genes in the aged than in the young condition, whereas the lymphatics-ablated group had the fewest in both directions (**Fig. 12b**). Although there are substantial age-associated changes, the overall microglia transcriptional response to peripheral IL-1β remains consistent with young mice, sharing approximately two-thirds of the top 25 significant differentially expressed genes with both sham and meningeal lymphatics ablation conditions (**Tables 1, 2, 11**). To identify aberrations in the microglial response to peripheral inflammation that were common to both the 'lymphatic ablation' and the 'aged' conditions, we identified differentially expressed genes and enriched biological-process pathways in the 'ablation' or 'aged' response compared to the 'adult (sham)' response to IL-1β, on a cluster-by-cluster basis (**Supplemental file**). The intersection of enriched biological pathways reveals modest overlap in shared pathways among

homeostatic microglia (cluster 0), under 29% of upregulated pathways and only 6% of downregulated pathways in meningeal lymphatics ablation cluster 0 are shared by the aged response (**Fig. 12c**). By contrast, we observe robust overlap in shared pathways among inflammation-associated microglia (cluster 2) between the meningeal lymphatics ablation and aged response to peripheral IL-1β, over 86% of upregulated biological pathways in meningeal lymphatics ablation inflammation-associated microglia are also observed in aged mice and while there are substantially more downregulated biological processes in meningeal lymphatics ablation than aged relative to sham, over half of downregulated pathways observed in the aged response to IL-1β are present in meningeal lymphatics ablation (**Fig. 12c**). A representative selection of upregulated pathways common to the 'ablation' and the 'aged' response to peripheral inflammation in inflammation-associated microglia (**Fig. 12d**) reveals that pathways related to inflammation and immune cell activation are elevated in both the experimental lymphatics ablation and aged response to peripheral IL-1β. This finding is consistent with our assumption that although broad microglia activation is impaired by meningeal lymphatic impairment, there may be compensatory hyperactivation of a subset of microglia. Paradoxically, we identify 25 pathways which are present in both lists of the shared 298 upregulated and the shared 66 downregulated pathways common to 'ablation' and 'aging' in inflammation-associated microglia (**Fig. 12e, Supplemental file**), indicating potential dysregulation resulting from meningeal lymphatic impairment relevant in aging.

Finally, we test whether experimental enhancement of meningeal lymphatic vessels can reduce the severity of sickness behavior symptoms in aged mice. Experimental treatment of meningeal lymphatic vessels by means of AAV-mediated overexpression of VEGFc can enhance the functioning of meningeal lymphatic vessels and improve glymphatic function in

aged mice which naturally exhibit more severe sickness behavior than young mice (Da Mesquita et al, 2018b; Godbout et al, 2008). Following AAV-VEGFc or AAV-LacZ control treatment in 2-year-old mice, we allowed 1 month for recovery and vector expression, which sufficed to increase lymphatic vessel diameter on average by over 7 μm (**Fig. 13a-c**). Notably, we found that such lymphatic enhancement sufficed to reduce the severity of sickness behavior in aged mice in response to peripheral IL-1β (**Fig. 13d**).

5.

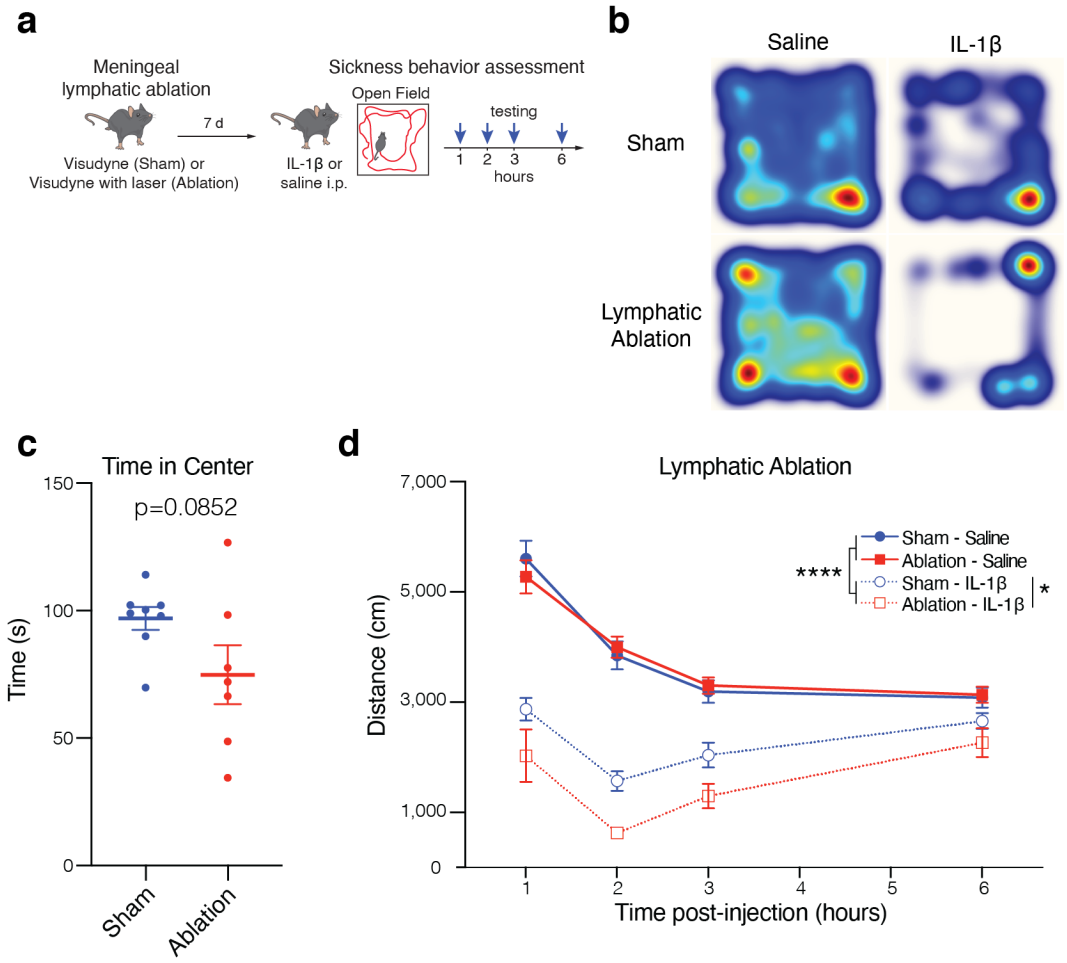

Figure 5. Meningeal lymphatic ablation exacerbates behavioral response to peripheral IL-1β. (**a**) Visudyne was injected i.c.m. and photoactivated for ablation or left without photoactivation for sham. IL-1β-induced sickness behavior was quantified by repeated 10-minute open field test at 1, 2, 3, and 6 hours following 1μg IL-1β or saline i.p. injection. (**b**) Representative heatmaps depict mouse position over 10 minutes, 2 hours following saline or IL-1β injection. (**c**) Cumulative time in center of arena comparing saline treated sham and ablation groups. (**d**) Locomotor activity of lymphatic ablated and sham mice following saline or IL-1β injection (n=6-8 mice per group). Data is represented as mean ± SEM; *p<0.05 ****p<0.0001; unpaired two-tailed t-test (**c**), three-way ANOVA (**d**). Representative of two independent experiments.

6.

a

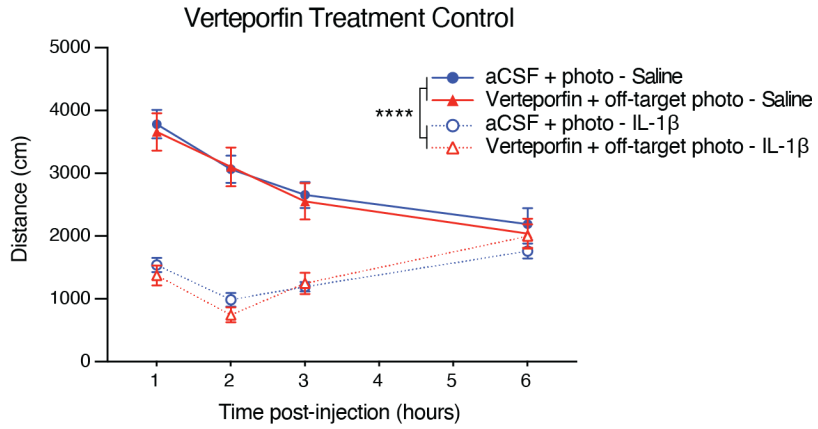

Figure 6. Off-target effects of verteporfin treatment do not contribute to behavioral response to peripheral IL-1β. Visudyne or aCSF control was injected i.c.m. and photoactivated at 5 sites, each ~1mm away from known lymphatic vessel locations. Following 1-week recovery, mice were injected i.p. with 1μg IL-1β or saline and subjected to repeated 10-minute open field test. Data is represented as mean ± SEM; n=6-8, ****p<0.0001; 2-way ANOVA.

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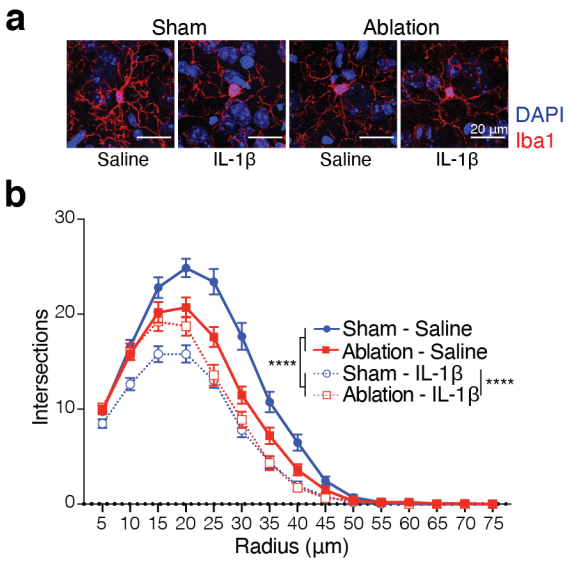

Figure 7. Meningeal lymphatic ablation impairs microglia morphological response to peripheral IL-1β. (**a**) Representative images of microglia (Iba1) immunostaining 2 hours following IL-1β or saline i.p. injection. (**b**) Sholl analysis of microglia complexity. Data is represented as mean ± SEM; n=45 cells from 3 mice per group, ****p<0.0001; three-way ANOVA.

8.

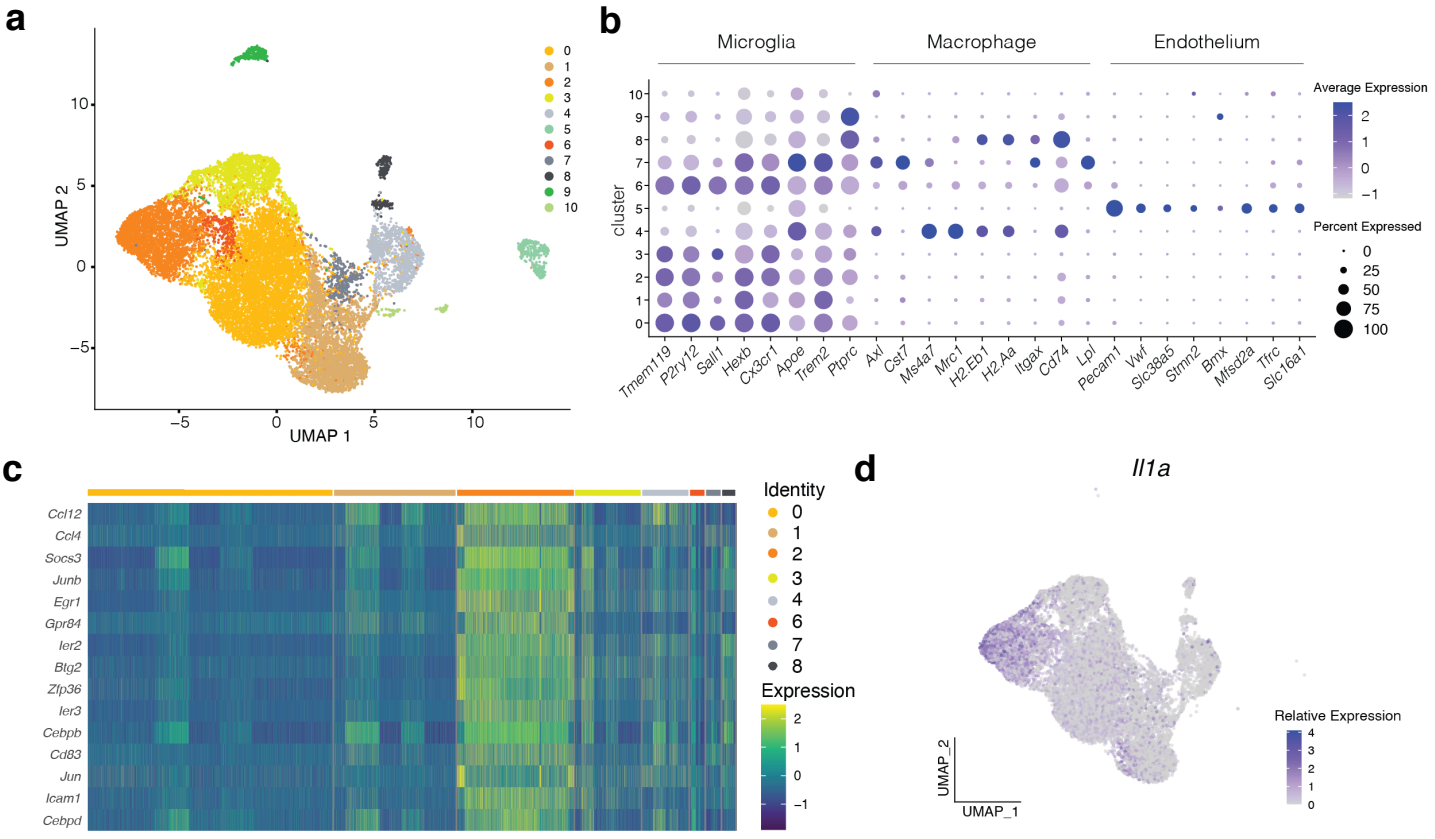

Figure 8. Single-cell sequencing of microglia, macrophages and endothelial cells after IL-1β induced sickness. (**a**) UMAP representation for brain CD11b^{+} cells highlighting the different cluster with and without lymphatic ablation and injected with saline or IL-1β. (**b**) Dot plot of cell type marker expression by cluster. (**c**) Heatmap of mean expression of genes used to define inflammatory microglia. (**d**) Features plot depicts the distribution of *Il1a*

9.

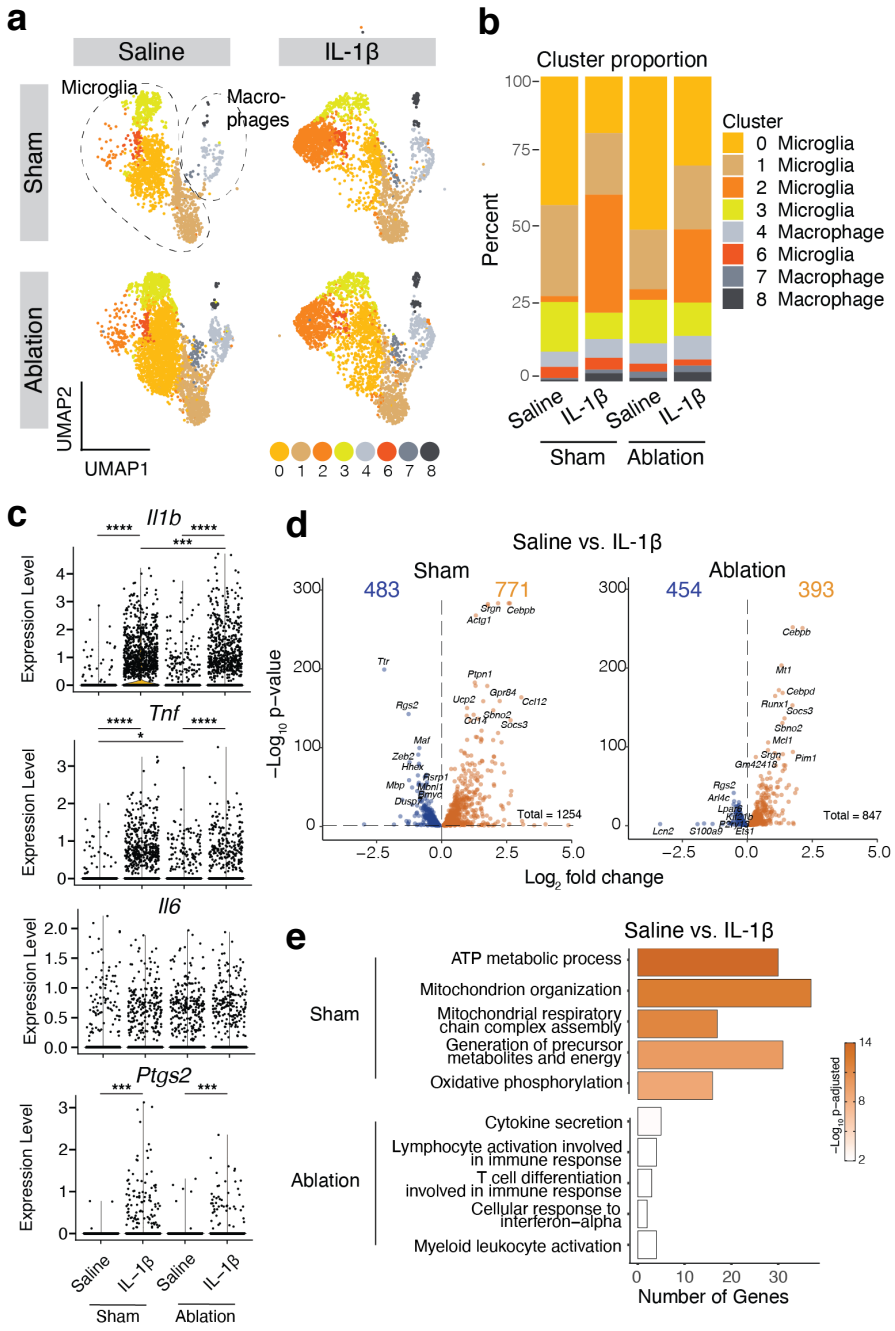

Figure 9. Meningeal lymphatic ablation impairs microglia activation and transcriptional response to peripheral inflammation. (**a-e**) Enrichment of CD11b+ cells from brains of ablation and sham mice, 2 hours after IL-1β or saline injection was achieved by magnetic-activated cell sorting. Transcriptomes were analyzed by single-cell RNA sequencing (n=5 mice per group). (**b**) UMAP representation of sequenced cells including microglia and macrophages with endothelial cell cluster removed. Different cell cluster are highlighted and split by experimental conditions. (**c**) Representation of cluster proportions for each condition. (**d**) Violin plots showing microglia expression of proinflammatory cytokines *Il1b, Tnf, Il6* and *Ptgs2*. (**e**) Volcano plots depict significantly down-regulated (blue) and up-regulated (orange) genes between saline and IL-1β treatments for sham and ablation mice. (**f**) Top 5 unique gene ontology terms enriched in sham (out of 302 total) and ablation (out of 42 total) in response to IL-1β by lowest adjusted p-value. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg (**c-e**).

10.

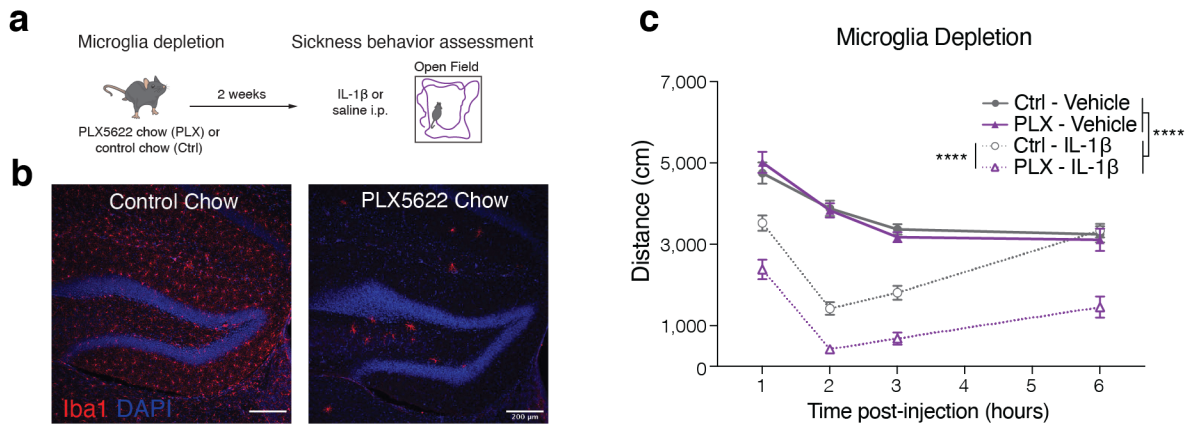

Figure 10. Microglia depletion exacerbates behavioral response to peripheral inflammation. (**a-c**) Microglia depletion strategy using PLX5622 chow. (**a**) Schematic, (**b**) representative confocal images of brain sections stained with DAPI and anti-Iba1, and (**c**) locomotor activity of microglia-depleted and control mice following IL-1β or saline i.p. injection (n=8 mice per group). Data is represented as mean ± SEM; ****p<0.0001 3-way AVOVA with Tukey post-hoc test in (**c**) Representative of two independent experiments.

11.

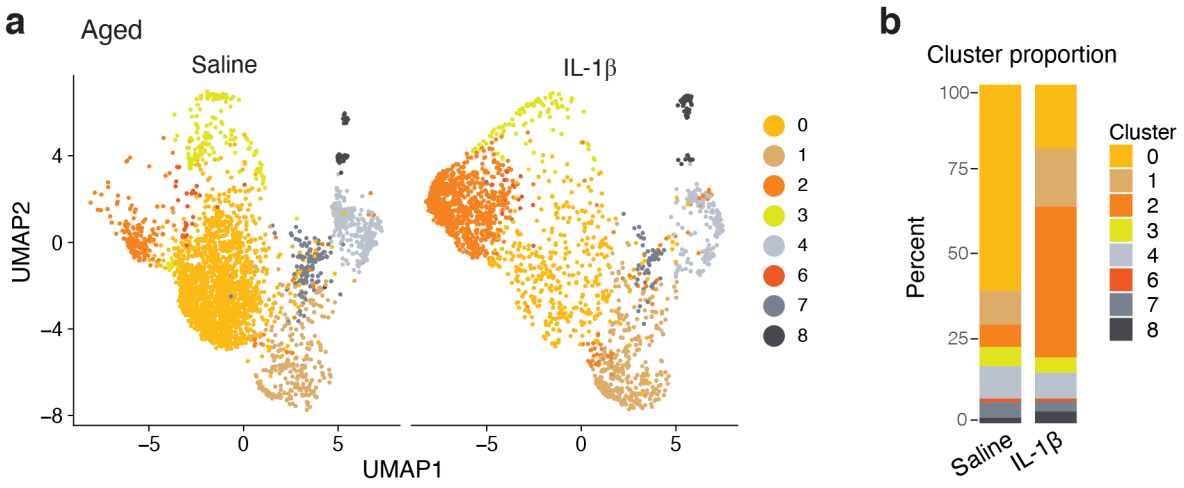

Figure 11. Single-cell sequencing of aged microglia and macrophages and after IL-1β induced sickness. (**a**) UMAP representation of CD11b^{+} sequenced cells from aged mice 2-hours following saline or IL-1β i.p. injection. (**b**) Cluster distribution compared between saline and IL-1β treatment.

12.

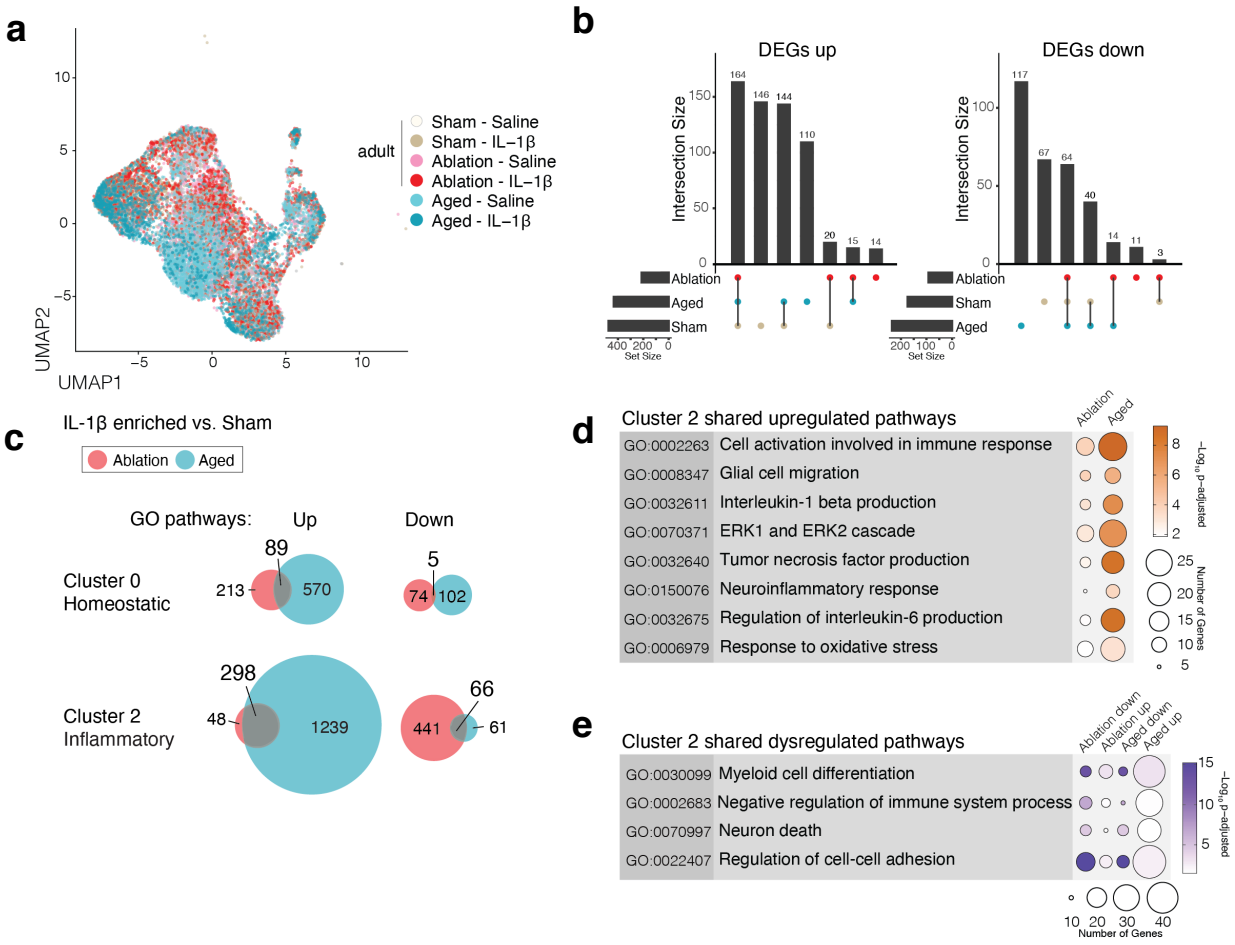

Figure 12. Enhancement of meningeal lymphatic function in aged mice reduces the severity of sickness behavior. (**a-e**) CD11b^{+} cells from aged mice were isolated by magnetic-activated cell sorting 2 hours following IL-1β or saline i.p. injection and sequenced (5 pooled per sample). (**a**) Aged mouse data were integrated into the adult mouse lymphatic ablation dataset for comparison and represented as UMAP depicting the distribution of cells across conditions. (**b**) Differentially expressed genes between saline and IL-1β treatments were compared for each condition (sham, ablation, aged), and the overlap in upregulated (left) and downregulated (right) genes is shown. (**c**) Gene ontology pathways in "Ablation - IL-1β" or "Aged - IL-1β" compared with adult control ("Sham - IL-1β"). Quantification of the overlap in altered pathways upregulated (left) and downregulated pathways (right) is shown for cluster 0 (homeostatic microglia) and cluster 2 (inflammation-associated microglia). (**d**) Selection of significantly elevated pathways in lymphatic ablation and aged cluster 2 microglia over adult control. (**e**) A representative selection of 25 potentially dysregulated pathways in cluster 2 that are both elevated and reduced in lymphatic ablation and aging compared to sham. The p-value and number of genes identified in each pathway is presented for each comparison. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg (**b-e**).

13.

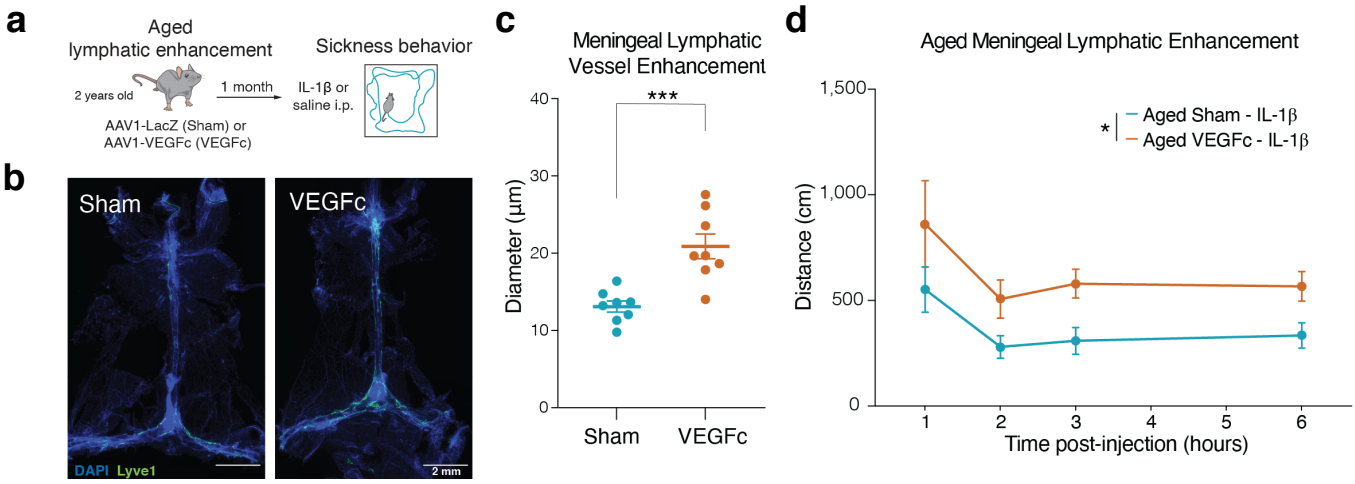

Figure 13. Enhancement of meningeal lymphatic function in aged mice reduces the severity of sickness behavior. (**a-c**) AAV1-LacZ (sham) or AAV1-VEGFc was administered to aged (~2-year-old) mice by i.c.m. injection to achieve meningeal lymphatic enhancement after 1-month incubation period. (**a**) Diagram showing timepoints of AAV administration and sickness behavior induction. (**b**) Representative confocal images of dural meninges stained with DAPI and anti-Lyve1 antibody. (**c**) Quantification of the average lymphatic vessel diameter after VEGFc treatment (n=8 mice per group). (**d**) Locomotor activity of lymphatic-enhanced and control mice following 0.5 µg IL-1β i.p. injection (n=8 mice per group). Data is represented as mean ± SEM; *p<0.05 ***p<0.001, unpaired two-tailed t test in (**c**), two-way ANOVA with Šidák post-hoc test in (**d**). Representative of two independent experiments in (**c**, **d**).

1.

gene_id	logFC	Pval	pct.1	pct.2
Cebpb	2.6215	0	96.44	53.47
Mt1	2.5770	0	87.34	41.97
Cebpd	2.1697	0	86.91	47.61
Srgn	1.7767	6.71E-283	82.89	52.28
Runx1	1.7697	2.05E-280	80.99	40.93
Actg1	1.3132	2.21E-268	94.15	76.38
Ttr	-2.2095	8.88E-200	10.07	51.36
Ptpn1	1.2737	2.74E-183	81.65	49.89
Mcl1	1.3026	3.13E-179	85.17	53.47
Zfand5	1.7530	5.61E-179	72.90	35.02
Ccl12	3.0617	1.40E-164	87.30	24.16
Gpr84	2.2313	7.43E-160	65.74	24.05
Fkbp5	1.5982	1.11E-159	66.12	20.79
Ucp2	0.9757	5.40E-151	87.11	62.11
Sbno2	1.9840	4.17E-148	65.20	14.50
Rgs2	-1.2723	2.12E-143	36.28	62.32
Cd14	1.2293	1.79E-142	83.66	60.10
Eif4a1	0.9643	3.09E-141	85.91	65.69
Bach1	1.4018	2.75E-137	67.40	34.53
Socs3	2.6502	1.47E-135	86.49	9.72
Btg1	1.1124	4.68E-135	83.20	54.72
C5ar1	1.5333	2.89E-122	62.25	24.81
Gcnt2	2.1681	2.22E-119	52.19	6.24
Irf2bpl	1.3140	2.06E-114	59.93	24.21
Rbpj	1.3630	6.33E-114	58.73	24.32

Table 1. Top 25 Differentially Expressed Genes in Sham Microglia Response to IL-1β. Differential gene expression comparing microglia of sham-IL-1β vs. sham-saline. Top 25 genes by p-value depicted with log fold change (logFC) and percent of cells with observed expression in sham-IL-1β (pct.1) and sham-saline (pct.2). F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

2.

gene_id	logFC	Pval	pct.1	pct.2
Cebpb	1.7446	0	86.12	58.50
Ccl12	2.1226	2.20E-251	72.99	46.42
Mt1	1.3134	5.31E-204	74.49	58.78
Cebpd	1.2128	1.20E-172	70.02	49.36
Fkbp5	1.3571	3.79E-169	52.24	19.98
Runx1	1.0703	2.87E-165	65.33	47.67
Socs3	1.7352	2.05E-153	61.93	17.53
Sbno2	1.4289	6.72E-137	43.90	20.37
Gpr84	1.3487	5.86E-131	51.99	38.45
Mcl1	0.8007	2.59E-106	75.78	59.85
Srgn	0.7931	1.38E-96	74.63	64.54
Pim1	1.7516	2.59E-94	31.91	7.94
Icam1	1.0532	8.98E-93	49.48	32.11
Gcnt2	1.3672	2.04E-91	33.17	10.41
Gm42418	0.3264	7.87E-88	100.00	100.00
Cd83	1.2046	5.51E-86	48.59	32.96
Map3k8	1.4073	1.75E-77	28.66	8.31
Jdp2	1.4605	3.70E-77	23.29	4.89
Slfn2	1.0939	4.52E-77	43.90	20.85
Arid5a	1.4356	7.90E-73	25.83	7.44
Rcan1	1.3603	3.78E-70	33.60	10.91
Zfand5	0.8421	7.76E-68	54.42	42.12
Ucp2	0.5219	1.28E-63	79.03	66.64
Il4ra	0.8924	5.00E-61	41.47	25.97
Irf2bpl	0.7604	3.17E-60	45.37	29.64

Table 2. Top 25 Differentially Expressed Genes in Meningeal Lymphatic Ablation Microglia Response to IL-1β. Differential gene expression comparing microglia of ablation-IL-1β vs. ablation-saline. Top 25 genes by p-value depicted with log fold change (logFC) and percent of cells with observed expression in ablation-IL-1β (pct.1) and ablation-saline (pct.2). F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

3.

gene_id	logFC	Pval	pct.2	pct.1
Ttr	-2.1367	2.17E-230	51.36	11.14
Mbp	-1.5589	4.22E-98	58.31	16.25
Dnaja1	-0.7712	1.05E-72	69.87	53.66
Apoe	1.1169	4.16E-45	68.62	86.72
Hsp90ab1	-0.4195	4.16E-45	91.04	90.34
Actg1	0.4564	3.17E-36	76.38	87.42
Gm47283	-0.6368	3.05E-35	44.84	35.03
Hsph1	-0.8846	5.98E-27	16.88	7.77
H2.D1	0.4295	6.53E-27	76.28	85.24
Rbm3	0.5689	4.17E-26	32.74	50.79
Cd63	0.4446	7.65E-26	68.73	79.68
H2.K1	0.4359	2.09E-23	66.34	78.55
Rack1	0.2818	5.38E-21	89.79	93.51
Cacybp	-0.4958	2.43E-20	38.17	31.54
Lyz2	0.5001	2.43E-20	67.16	77.05
Eef1a1	0.2493	8.81E-19	98.32	98.25
Cd52	0.3865	1.20E-18	70.36	78.80
Son	0.2677	1.64E-17	76.28	86.62
Srsf5	0.3508	1.84E-17	52.23	72.83
Cd9	0.2801	2.93E-17	86.21	91.36
Evi2a	0.3407	4.68E-17	55.92	69.76
Mobp	-0.9754	5.21E-17	26.11	3.42
Fau	0.2311	6.60E-17	97.88	98.80
Tmem86a	0.3402	2.91E-16	50.43	66.44
P4ha1	-0.3591	3.71E-16	50.38	47.42

Table 3. Top 25 Differentially Expressed Genes in Meningeal Lymphatic Ablation vs. Sham Microglia Following Saline. Differential gene expression comparing microglia of ablation-saline vs. sham-saline. Top 25 genes by p-value depicted with log fold change (logFC) and percent of cells with observed expression in ablation-saline (pct.1) and sham-saline (pct.2). F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

4.

gene_id	logFC	Pval	pct.1	pct.2
Cebpd	-1.0244	4.20E-146	86.91	70.02
Cebpb	-0.8561	6.38E-144	96.44	86.12
Hsp90ab1	-0.5902	9.10E-90	94.54	91.63
Mt1	-0.8373	1.53E-88	87.34	74.49
Ptpn1	-0.6826	1.36E-85	81.65	68.73
Runx1	-0.6777	3.12E-85	80.99	65.33
Srgn	-0.7464	3.96E-85	82.89	74.63
Zfand5	-0.8036	9.67E-71	72.90	54.42
Bach1	-0.6784	2.37E-55	67.40	49.23
Picalm	-0.4864	3.56E-55	89.28	82.18
Actg1	-0.4603	7.47E-50	94.15	91.88
Eif4a1	-0.4652	5.73E-48	85.91	80.36
Eif1a	-0.6943	1.99E-46	53.39	35.89
Ucp2	-0.4431	2.71E-46	87.11	79.03
Rgs2	0.7101	3.50E-46	36.28	50.48
C5ar1	-0.6687	4.66E-46	62.25	45.01
B4galt1	-0.5392	7.41E-44	65.12	49.34
Ldha	-0.5969	5.73E-43	63.65	50.88
Cd14	-0.5358	2.22E-41	83.66	76.24
Rbpj	-0.5930	1.83E-39	58.73	43.61
Mcl1	-0.4559	9.61E-38	85.17	75.78
Samsn1	-0.4955	1.19E-37	69.26	57.35
Skil	-0.4530	2.69E-37	75.61	66.76
Rcan1	-0.7201	1.14E-36	59.66	33.60
Hhex	0.7823	2.04E-35	16.61	29.41
Irf2bp2	-0.4283	3.57E-35	74.33	63.86

Table 4. Top 25 Differentially Expressed Genes in Meningeal Lymphatic Ablation vs. Sham Microglia responding to IL-1β. Differential gene expression comparing microglia of ablation-IL-1β vs. sham- IL-1β. Top 25 genes by p-value depicted with log fold change (logFC) and percent of cells with observed expression in ablation-IL-1β (pct.1) and sham- IL-1β (pct.2). F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

5.

ID	Description	pvalue	p.adjust	qvalue	geneID	Count
GO:0007623	circadian rhythm	5.70E-07	1.29E-03	1.03E-03	Rock2/Ptgds/Nrip1/Kdm2a/Creb1/Mat2a/Zfhx3/Top1/Sfpq	9
GO:0071826	ribonucleoprotein complex subunit organization	2.50E-06	2.83E-03	2.25E-03	Ptges3/Celf2/Hsp90aa1/Hsp90ab1/Cpsf6/Kif5b/Mbnl1/Prpf39	8
GO:0051648	vesicle localization	6.91E-06	4.05E-03	3.22E-03	Madd/Kif5b/Picalm/Mlph/Tcf7l2/Kif1b/Dpysl2	7
GO:0006457	protein folding	7.53E-06	4.05E-03	3.22E-03	Dnaja1/Ptges3/Hsph1/Hsp90aa1/Hsp90ab1/Hspd1/St13	7
GO:0048511	rhythmic process	8.95E-06	4.05E-03	3.22E-03	Rock2/Ptgds/Nrip1/Kdm2a/Creb1/Mat2a/Zfhx3/Top1/Sfpq	9
GO:0022618	ribonucleoprotein complex assembly	2.02E-05	6.18E-03	4.92E-03	Ptges3/Celf2/Hsp90aa1/Hsp90ab1/Cpsf6/Mbnl1/Prpf39	7
GO:0042752	regulation of circadian rhythm	2.30E-05	6.18E-03	4.92E-03	Rock2/Ptgds/Kdm2a/Creb1/Zfhx3/Sfpq	6
GO:0006458	de novo' protein folding	2.33E-05	6.18E-03	4.92E-03	Ptges3/Hsph1/Hspd1/St13	4
GO:1903311	regulation of mRNA metabolic process	2.46E-05	6.18E-03	4.92E-03	Rock2/Celf2/Cpsf6/Khdrbs3/Mbnl1/Larp1/Tra2a/Srsf7	8
GO:0048588	developmental cell growth	2.98E-05	6.76E-03	5.38E-03	Zeb2/Hsp90aa1/Hsp90ab1/Picalm/Plxna4/Jade2/Kmt2d/Dpysl2	8
GO:0021955	central nervous system neuron axonogenesis	3.93E-05	8.08E-03	6.43E-03	Zeb2/Hsp90aa1/Hsp90ab1/Plxna4	4
GO:0051131	chaperone-mediated protein complex assembly	4.28E-05	8.08E-03	6.43E-03	Ptges3/Hsp90aa1/Hsp90ab1	3
GO:0032252	secretory granule localization	5.25E-05	8.50E-03	6.76E-03	Kif5b/Tcf7l2/Kif1b	3
GO:1900037	regulation of cellular response to hypoxia	5.25E-05	8.50E-03	6.76E-03	Rock2/Pink1/Chchd2	3
GO:0050684	regulation of mRNA processing	5.85E-05	8.83E-03	7.02E-03	Celf2/Cpsf6/Khdrbs3/Mbnl1/Tra2a/Srsf7	6
GO:0072655	establishment of protein localization to mitochondrion	6.38E-05	9.03E-03	7.18E-03	Dnaja1/Hsph1/Hsp90aa1/Pink1/Hspd1	5
GO:0070585	protein localization to mitochondrion	8.26E-05	1.10E-02	8.75E-03	Dnaja1/Hsph1/Hsp90aa1/Pink1/Hspd1	5
GO:1903320	regulation of protein modification by small protein conjugation or removal	9.95E-05	1.20E-02	9.52E-03	Ivns1abp/Dnaja1/Hsp90aa1/Hsp90ab1/Pink1/Kdm2a/Capn3	7
GO:0032755	positive regulation of interleukin-6 production	1.00E-04	1.20E-02	9.52E-03	Il6ra/Mbp/Tlr3/Hspd1/Tlr7	5
GO:0048024	regulation of mRNA splicing, via spliceosome	1.33E-04	1.50E-02	1.19E-02	Celf2/Khdrbs3/Mbnl1/Tra2a/Srsf7	5

Table 5. Top 20 Downregulated Biological Pathways in Meningeal Lymphatic Ablation Microglia. Enriched downregulated biological pathways based on differentially expressed genes between ablation-saline and sham-saline microglia. Top 20 by adjusted p-value depicted. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

6.

ID	Description	pvalue	p.adjust	qvalue	geneID	Count
GO:0046034	ATP metabolic process	6.65E-19	2.86E-15	1.91E-15	Uqcrq/Cdk1/Uqcrh/Tpi1/Atp5c1/Hif1a/Ndufa7/Gpi1/Atp5k/Cox7c/Cox4i1/Uqcr10/Mif/Ier3/Ndufs6/Park7/Pgk1/Bcl2l1/Ndufv3/Gapdh/Atpif1/Cox5a/Atp5e/Cox5b/Cox7a2l/Atp5g2/Atp5j/Atp5l/Igf1/Pkm/Tspo/Atp5d	32
GO:0001819	positive regulation of cytokine production	7.77E-13	1.67E-09	1.12E-09	Ncl/Cd83/Ccl2/Mapkapk2/Cd74/Il1a/Nop53/Lpl/Cybb/Irf7/Hif1a/Ccr2/Egr1/Ccdc88b/Serpine1/Irf8/Mif/Tnf/Park7/C3ar1/Hmgb2/Ptger4/Fgr/Gapdh/Ifi204/Slc11a1/Crlf2/Ccl5/Casp4/Lrp1/Ly9/Cd84/Tlr2/C3	34
GO:0044403	symbiotic process	2.18E-12	3.12E-09	2.08E-09	Eif3f/Hpn/Ifitm2/Axl/Ifitm3/Cd74/Zfp36/Fam111a/Top2a/Banf1/Cfl1/Jun/Tnf/Bcl2l1/Isg15/Gapdh/Eea1/Bst2/Apobec3/Ddx3x/Fn1/Zbp1/Ccl5/Lgals1/Camp/Denr/Tlr2/Apoe/Gbp2	29
GO:0032103	positive regulation of response to external stimulus	5.53E-12	5.96E-09	3.97E-09	Ccl2/Cd74/Calr/S100a8/S100a9/Lpl/Cd180/Nfkbia/Lag3/Irf7/Ccr1/Ccr2/Serpine1/Mif/Tnf/Park7/C3ar1/Hmgb2/Ptger4/Cxcl10/Ifi204/Ifi203/Fn1/Zbp1/Ccl5/P2rx4/Nrp1/Lrp1/Tlr2/C3/Rac2	31
GO:0097529	myeloid leukocyte migration	1.47E-11	1.27E-08	8.46E-09	Ccl2/Ccl12/Cd9/Cd74/S100a8/S100a9/Il1a/Spp1/Ccr1/Ccr2/Serpine1/Mif/Slamf8/C3ar1/Cxcl2/Ptger4/Cxcl10/Ccl5/P2rx4/Ccl9/Rac2/Cx3cr1	22
GO:0002237	response to molecule of bacterial origin	2.74E-11	1.96E-08	1.31E-08	Ncl/Mef2c/Ccl2/Mapkapk2/Ccl12/Axl/Zfp36/Cd180/Junb/Nfkbia/Jun/Serpine1/Irf8/Mif/Jund/Tnf/Hmgb2/Cxcl2/Ptger4/Cxcl10/Cxcl16/Fcgr2b/Fcgr4/Slc11a1/Ccl5/Cd84/Tlr2/Gbp2/Cx3cr1	29
GO:0050900	leukocyte migration	6.57E-11	4.04E-08	2.69E-08	Ccl2/Ccl12/Cd9/Cd74/Calr/S100a8/S100a9/Il1a/Spp1/Ccr1/Ccr2/Serpine1/Mif/Tnf/Slamf8/C3ar1/Cxcl2/Ptger4/Cxcl10/Cxcl16/Ccl5/P2rx4/Ccl9/Itga6/Tlr2/Rac2/Cx3cr1	27
GO:0060326	cell chemotaxis	1.14E-10	6.15E-08	4.10E-08	Ccl2/Ccl12/Saa3/Cd74/Calr/S100a8/S100a9/Spp1/Ccr1/Ccr2/Serpine1/Mif/Slamf8/C3ar1/Hmgb2/Cxcl2/Cxcl10/Cxcl16/Ccl5/P2rx4/Ccl9/Nrp1/Ccrl2/Rac2/Cx3cr1	25
GO:0016032	viral process	2.03E-10	8.98E-08	5.99E-08	Eif3f/Hpn/Ifitm2/Axl/Ifitm3/Cd74/Zfp36/Fam111a/Top2a/Banf1/Cfl1/Jun/Tnf/Bcl2l1/Isg15/Eea1/Bst2/Apobec3/Ddx3x/Zbp1/Ccl5/Lgals1/Denr/Apoe	24
GO:0032496	response to lipopolysaccharide	2.23E-10	8.98E-08	5.99E-08	Ncl/Mef2c/Ccl2/Mapkapk2/Ccl12/Axl/Zfp36/Cd180/Junb/Nfkbia/Jun/Serpine1/Irf8/Mif/Jund/Tnf/Hmgb2/Cxcl2/Ptger4/Cxcl10/Cxcl16/Fcgr4/Slc11a1/Ccl5/Cd84/Gbp2/Cx3cr1	27
GO:0042775	mitochondrial ATP synthesis coupled electron transport	2.30E-10	8.98E-08	5.99E-08	Uqcrq/Cdk1/Uqcrh/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Cox5a/Cox5b	12
GO:0034341	response to interferon-gamma	2.77E-10	9.32E-08	6.22E-08	Ccl2/Ccl12/Ifitm2/Ifitm3/Actg1/Irf8/Rab20/Cxcl16/Gapdh/Bst2/Slc11a1/Capg/Ccl5/Ccl9/Epsr/Tlr2/Gbp2	17
GO:0006091	generation of precursor metabolites and energy	2.82E-10	9.32E-08	6.22E-08	Uqcrq/Cdk1/Uqcrh/Tpi1/Nop53/Taldo1/Hif1a/Ndufa7/Gpi1/Cox7c/Cox4i1/Uqcr10/Mif/Ier3/Ndufs6/Park7/Pgk1/Ndufv3/Gapdh/Cox5a/Cox5b/Pgls/Cox7a2l/Ppp1r3d/Gnas/Igf1/Pkm/Atp5d	28
GO:0042773	ATP synthesis coupled electron transport	5.31E-10	1.63E-07	1.09E-07	Uqcrq/Cdk1/Uqcrh/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Cox5a/Cox5b	12
GO:0030595	leukocyte chemotaxis	9.71E-10	2.79E-07	1.86E-07	Ccl2/Ccl12/Cd74/Calr/S100a8/S100a9/Spp1/Ccr1/Ccr2/Serpine1/Mif/Slamf8/C3ar1/Cxcl2/Cxcl10/Cxcl16/Ccl5/Ccl9/Rac2/Cx3cr1	20
GO:0006119	oxidative phosphorylation	1.41E-09	3.80E-07	2.53E-07	Uqcrq/Cdk1/Uqcrh/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Pgk1/Ndufv3/Cox5a/Cox5b/Cox7a2l	14
GO:0050727	regulation of inflammatory response	1.68E-09	4.26E-07	2.84E-07	Cst7/S100a8/S100a9/Zfp36/Lpl/Socs3/Nfkbia/Ccr2/Pmp22/Serpine1/Mif/Ier3/Tnf/Slamf8/Park7/Ptger4/Fcgr2b/Adcy7/Ccl5/Gpx4/Igf1/Tlr2/C3/Apoe/Cx3cr1	25
GO:0043903	regulation of interspecies interactions between organisms	2.08E-09	4.98E-07	3.32E-07	Hpn/Ifitm2/Ifitm3/Cd74/Zfp36/Fam111a/Top2a/Banf1/Cfl1/Jun/Tnf/Isg15/Gapdh/Bst2/Apobec3/Ddx3x/Ccl5/Lgals1/Apoe	19
GO:1990266	neutrophil migration	2.51E-09	5.70E-07	3.80E-07	Ccl2/Ccl12/Cd74/S100a8/S100a9/Il1a/Spp1/Slamf8/C3ar1/Cxcl2/Ptger4/Cxcl10/Ccl5/Ccl9/Rac2	15
GO:0015985	energy coupled proton transport, down electrochemical gradient	3.07E-09	6.29E-07	4.19E-07	Atp5c1/Atp5k/Atp5e/Cox5b/Atp5g2/Atp5j/Atp5l/Atp5d	8

Table 6. Top 20 Upregulated Biological Pathways in Meningeal Lymphatic Ablation Microglia. Enriched upregulated biological pathways based on differentially expressed genes between ablation-saline and sham-saline microglia. Top 20 by adjusted p-value depicted. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

7.

ID	Description	pvalue	p.adjust	qvalue	geneID	Count
GO:0042326	negative regulation of phosphorylation	5.92E-06	7.50E-03	6.21E-03	Zmynd11/Dnaja1/Igf1r/Hsph1/Atxn1/Gsk3b/Ctdsp2/Dynll1/Prkcd/Cdkn1c/Cbl/Dusp6/Prkar2a	13
GO:0043434	response to peptide hormone	6.80E-06	7.50E-03	6.21E-03	Igf1r/Gkap1/Cacybp/Gdf15/Gsk3b/Creb1/Prkcb/Prkcd/Stat1/Atp2b1/Lpin2	11
GO:0035264	multicellular organism growth	8.19E-06	7.50E-03	6.21E-03	Nipbl/Celf1/Rc3h2/Gdf15/Kdm2a/Creb1/Dicer1/Cdkn1c/Ncoa3	9
GO:0048511	rhythmic process	1.62E-05	1.12E-02	9.24E-03	Setx/Igf1r/Rbm4b/Crebbp/Ptgds/Nrip1/Gsk3b/Kdm2a/Creb1/Kmt2a	10
GO:0071375	cellular response to peptide hormone stimulus	2.51E-05	1.13E-02	9.33E-03	Igf1r/Gkap1/Gdf15/Gsk3b/Prkcb/Prkcd/Stat1/Atp2b1/Lpin2	9
GO:0032869	cellular response to insulin stimulus	3.13E-05	1.13E-02	9.33E-03	Igf1r/Gkap1/Gsk3b/Prkcb/Prkcd/Stat1/Atp2b1/Lpin2	8
GO:0048872	homeostasis of number of cells	3.20E-05	1.13E-02	9.33E-03	Mertk/Kmt2e/Inpp5d/Rc3h2/Pik3cd/Tnfsf13b/Tcirg1/Arid4a/Stat1/Kmt2a	10
GO:1901652	response to peptide	3.28E-05	1.13E-02	9.33E-03	Igf1r/Gkap1/Cacybp/Gdf15/Gsk3b/Creb1/Prkcb/Prkcd/Stat1/Atp2b1/Lpin2	11
GO:0007623	circadian rhythm	5.32E-05	1.62E-02	1.34E-02	Setx/Rbm4b/Ptgds/Nrip1/Gsk3b/Kdm2a/Creb1/Kmt2a	8
GO:0033690	positive regulation of osteoblast proliferation	8.44E-05	1.95E-02	1.61E-02	Igf1r/Gsk3b/Itgav	3
GO:1901653	cellular response to peptide	8.79E-05	1.95E-02	1.61E-02	Igf1r/Gkap1/Gdf15/Gsk3b/Prkcb/Prkcd/Stat1/Atp2b1/Lpin2	9
GO:0006650	glycerophospholipid metabolic process	9.22E-05	1.95E-02	1.61E-02	Pnpla8/Inpp5d/Smg1/Pik3cd/Fam126b/Lpcat1/Cds1/Etnk1	8
GO:0032868	response to insulin	9.22E-05	1.95E-02	1.61E-02	Igf1r/Gkap1/Gsk3b/Prkcb/Prkcd/Stat1/Atp2b1/Lpin2	8
GO:0043409	negative regulation of MAPK cascade	1.07E-04	2.10E-02	1.74E-02	Zmynd11/Dnaja1/Igf1r/Hsph1/Gsk3b/Prkcd/Dusp6	7
GO:0048608	reproductive structure development	1.32E-04	2.37E-02	1.96E-02	Mertk/Nipbl/Igf1r/Nrip1/Arid1a/Dicer1/Itgav/Bptf/Arid4a/Cdkn1c/Ncoa3	11
GO:0061458	reproductive system development	1.43E-04	2.37E-02	1.96E-02	Mertk/Nipbl/Igf1r/Nrip1/Arid1a/Dicer1/Itgav/Bptf/Arid4a/Cdkn1c/Ncoa3	11
GO:0035729	cellular response to hepatocyte growth factor stimulus	1.55E-04	2.37E-02	1.96E-02	Crebbp/Gsk3b/Creb1	3
GO:0080182	histone H3-K4 trimethylation	1.55E-04	2.37E-02	1.96E-02	Kmt2e/Arid4a/Kmt2a	3
GO:0018023	peptidyl-lysine trimethylation	1.83E-04	2.56E-02	2.12E-02	Kmt2e/Kmt5b/Arid4a/Kmt2a	4
GO:0018105	peptidyl-serine phosphorylation	1.90E-04	2.56E-02	2.12E-02	Rptor/Rictor/Smg1/Clk1/Fnip1/Gsk3b/Prkcb/Prkcd/Mark2	9

Table 7. Top 20 Unique Downregulated Biological Pathways in Sham Microglia IL-1β Response. Enriched downregulated biological pathways based on differentially expressed genes between sham-saline and sham-IL-1β microglia. Top 20 by adjusted p-value depicted. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

8.

ID	Description	pvalue	p.adjust	qvalue	geneID	Count
GO:0046034	ATP metabolic process	6.28E-18	2.38E-14	1.89E-14	Uqcrq/Uqcrh/Uqcrb/Atp5j2/Pgam1/Atp5c1/Hif1a/Ndufa7/Aldoa/Cox7c/Cox4i1/Uqcr10/Mif/Ndufs6/Uqcc2/Atp5g1/Park7/Pgk1/Ndufv3/Gapdh/Atpif1/Iscu/Cox5a/Cox5b/Cox7a2/Atp5j/Atp5l/Igf1/Pkm/Atp5d	30
GO:0007005	mitochondrion organization	6.26E-16	1.19E-12	9.41E-13	Slc25a4/Hspa4/Ndufb11/Ndufa2/Sqstm1/Pet100/Uqcrb/Ndufa13/Ndufaf8/Hsp90aa1/Slirp/Ndufs5/Ndufa5/Hif1a/Pdcd5/Prelid1/Ndufc1/Ndufb5/Uqcr10/Ndufa1/Ndufb2/Ndufs6/Uqcc2/Timm10/Park7/Siva1/Ndufb7/Atpif1/Romo1/Chchd2/Cox20/Ndufa11/Fundc2/Cox7a2/Gabarap/Igf1/Atp5d	37
GO:0033108	mitochondrial respiratory chain complex assembly	4.72E-15	5.96E-12	4.73E-12	Ndufb11/Ndufa2/Pet100/Uqcrb/Ndufa13/Ndufaf8/Ndufs5/Ndufa5/Ndufc1/Ndufb5/Uqcr10/Ndufa1/Ndufb2/Uqcc2/Ndufb7/Cox20/Ndufa11	17
GO:0006091	generation of precursor metabolites and energy	2.89E-13	2.74E-10	2.17E-10	Uqcrq/Ptges3/Uqcrh/Uqcrb/Nop53/Pgam1/Taldo1/Ndufa5/Hif1a/Ndufa7/Prelid1/Aldoa/Cox7c/Cox4i1/Uqcr10/Mif/Ndufs6/Uqcc2/Park7/Pgk1/Ndufv3/Gapdh/Iscu/Cox5a/Cox5b/Cox20/Gnas/Cox7a2/Igf1/Pkm/Atp5d	31
GO:0006119	oxidative phosphorylation	3.64E-12	2.45E-09	1.95E-09	Uqcrq/Uqcrh/Uqcrb/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Uqcc2/Park7/Pgk1/Ndufv3/Iscu/Cox5a/Cox5b/Cox7a2	16
GO:0010257	NADH dehydrogenase complex assembly	4.58E-12	2.45E-09	1.95E-09	Ndufb11/Ndufa2/Ndufa13/Ndufaf8/Ndufs5/Ndufa5/Ndufc1/Ndufb5/Ndufa1/Ndufb2/Ndufb7/Ndufa11	12
GO:0032981	mitochondrial respiratory chain complex I assembly	4.58E-12	2.45E-09	1.95E-09	Ndufb11/Ndufa2/Ndufa13/Ndufaf8/Ndufs5/Ndufa5/Ndufc1/Ndufb5/Ndufa1/Ndufb2/Ndufb7/Ndufa11	12
GO:0042775	mitochondrial ATP synthesis coupled electron transport	5.18E-12	2.45E-09	1.95E-09	Uqcrq/Uqcrh/Uqcrb/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Iscu/Cox5a/Cox5b	13
GO:0042773	ATP synthesis coupled electron transport	1.31E-11	5.52E-09	4.38E-09	Uqcrq/Uqcrh/Uqcrb/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Iscu/Cox5a/Cox5b	13
GO:0045333	cellular respiration	2.20E-11	8.32E-09	6.60E-09	Uqcrq/Uqcrh/Uqcrb/Nop53/Ndufa5/Hif1a/Ndufa7/Prelid1/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Iscu/Cox5a/Cox5b/Cox20/Atp5d	19
GO:0022904	respiratory electron transport chain	4.29E-11	1.48E-08	1.17E-08	Uqcrq/Uqcrh/Uqcrb/Ndufa5/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Iscu/Cox5a/Cox5b	14
GO:0022900	electron transport chain	8.43E-11	2.66E-08	2.11E-08	Uqcrq/Uqcrh/Uqcrb/Ndufa5/Ndufa7/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Iscu/Cox5a/Cox5b	14
GO:0015980	energy derivation by oxidation of organic compounds	1.97E-10	5.76E-08	4.56E-08	Uqcrq/Ptges3/Uqcrh/Uqcrb/Nop53/Ndufa5/Hif1a/Ndufa7/Prelid1/Cox7c/Cox4i1/Uqcr10/Ndufs6/Park7/Ndufv3/Iscu/Cox5a/Cox5b/Cox20/Gnas/Igf1/Atp5d	22
GO:0009141	nucleoside triphosphate metabolic process	2.51E-09	6.79E-07	5.38E-07	Nme1/Atp5j2/Atp5c1/Aldoa/Dctpp1/Atp5g1/Smpdl3a/Ran/Cox5b/Atp5j/Cmpk1/Atp5l/Pkm/Atp5d	14
GO:0008380	RNA splicing	6.71E-09	1.56E-06	1.24E-06	Son/Lsm6/Nsrp1/Srrm2/Lsm2/Npm1/Hnrnph1/Sf3b3/Srsf5/Lsm5/Pabpc1/Srsf2/Luc7l2/Snrpf/Snrpd2/Lsm4/Prpf40a/C1qbp/Ubl5/Ybx1/Snrpe/Zcrb1/Hnrnpa1/Sfpq	24
GO:0000375	RNA splicing, via transesterification reactions	7.40E-09	1.56E-06	1.24E-06	Son/Lsm6/Nsrp1/Lsm2/Npm1/Sf3b3/Srsf5/Lsm5/Srsf2/Luc7l2/Snrpf/Snrpd2/Lsm4/Prpf40a/C1qbp/Ubl5/Snrpe/Zcrb1/Hnrnpa1/Sfpq	20
GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	7.40E-09	1.56E-06	1.24E-06	Son/Lsm6/Nsrp1/Lsm2/Npm1/Sf3b3/Srsf5/Lsm5/Srsf2/Luc7l2/Snrpf/Snrpd2/Lsm4/Prpf40a/C1qbp/Ubl5/Snrpe/Zcrb1/Hnrnpa1/Sfpq	20
GO:0000398	mRNA splicing, via spliceosome	7.40E-09	1.56E-06	1.24E-06	Son/Lsm6/Nsrp1/Lsm2/Npm1/Sf3b3/Srsf5/Lsm5/Srsf2/Luc7l2/Snrpf/Snrpd2/Lsm4/Prpf40a/C1qbp/Ubl5/Snrpe/Zcrb1/Hnrnpa1/Sfpq	20
GO:0006753	nucleoside phosphate metabolic process	8.53E-09	1.70E-06	1.35E-06	Nme1/Hprt/Atp5j2/Pgam1/Atp5c1/Hif1a/Aldoa/Hint1/Aprt/Mif/Dctpp1/Atp5g1/Adssl1/Park7/Pgk1/Smpdl3a/Nadk/Gapdh/Ran/Cox5b/Atp5j/Cmpk1/Atp5l/Igf1/Pkm/Atp5d	26
GO:0006413	translational initiation	1.31E-08	2.48E-06	1.97E-06	Eif3a/Eif5b/Eif3f/Npm1/Eif3m/Eif3e/Eif3k/Tpr/Eif3i/Ddx3x/Eif3h/Eif1ax/Eif5	13

Table 8. Top 20 Unique Upregulated Biological Pathways in Sham Microglia IL-1β Response. Enriched upregulated biological pathways based on differentially expressed genes between sham-saline and sham-IL-1β microglia. Top 20 by adjusted p-value depicted. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

9.

ID	Description	pvalue	p.adjust	qvalue	geneID	Count
GO:0050729	positive regulation of inflammatory response	1.21E-05	1.70E-02	1.49E-02	S100a8/S100a9/Lpl/Ets1/Serpine1/Park7/Ccl5	7
GO:0006119	oxidative phosphorylation	1.43E-05	1.70E-02	1.49E-02	Pink1/Cox4i1/Ndufs6/Park7/Cox7a2l/Mlxipl	6
GO:0002082	regulation of oxidative phosphorylation	2.91E-05	2.12E-02	1.86E-02	Pink1/Park7/Cox7a2l/Mlxipl	4
GO:0007005	mitochondrion organization	3.56E-05	2.12E-02	1.86E-02	Ndufb10/Timm13/Pink1/Ndufb5/Map1lc3a/Ndufs6/Park7/Ndufb7/Atpif1/Ndufa6/Cox7a2l	11
GO:0006091	generation of precursor metabolites and energy	5.00E-05	2.22E-02	1.94E-02	Oxct1/Tpi1/Pink1/Cox4i1/Ndufs6/Park7/Pgls/Cox7a2l/Mlxipl/Gnas	10
GO:0046034	ATP metabolic process	5.59E-05	2.22E-02	1.94E-02	Tpi1/Pink1/Cox4i1/Ndufs6/Park7/Atpif1/Cox7a2l/Mlxipl	8
GO:0032386	regulation of intracellular transport	9.42E-05	2.40E-02	2.10E-02	Cdk5r1/Cep290/Pcm1/Numa1/Pink1/Use1/Park7/Scp2/Atpif1	9
GO:0010257	NADH dehydrogenase complex assembly	1.01E-04	2.40E-02	2.10E-02	Ndufb10/Ndufb5/Ndufb7/Ndufa6	4
GO:0032981	mitochondrial respiratory chain complex I assembly	1.01E-04	2.40E-02	2.10E-02	Ndufb10/Ndufb5/Ndufb7/Ndufa6	4
GO:0031349	positive regulation of defense response	1.16E-04	2.40E-02	2.10E-02	S100a8/S100a9/Lpl/Ets1/Serpine1/Ankrd17/Park7/Ccl5	8
GO:0045787	positive regulation of cell cycle	1.17E-04	2.40E-02	2.10E-02	Cdk5r1/Numa1/Hsp90ab1/Tgfa/Ccnd1/Ankrd17/Anapc11/Ddx3x/Mlxipl	9
GO:1903578	regulation of ATP metabolic process	1.21E-04	2.40E-02	2.10E-02	Pink1/Park7/Atpif1/Cox7a2l/Mlxipl	5
GO:0071157	negative regulation of cell cycle arrest	1.63E-04	2.98E-02	2.61E-02	Hsp90ab1/Ccnd1/Mlxipl	3
GO:0006120	mitochondrial electron transport, NADH to ubiquinone	1.89E-04	3.21E-02	2.81E-02	Pink1/Ndufs6/Park7	3
GO:0030593	neutrophil chemotaxis	2.09E-04	3.32E-02	2.90E-02	S100a8/S100a9/Spp1/Cxcl2/Ccl5	5
GO:0006914	autophagy	2.49E-04	3.36E-02	2.94E-02	S100a8/S100a9/Rock1/Pink1/Rragc/Dap/Map1lc3a/Park7/Atpif1	9
GO:0061919	process utilizing autophagic mechanism	2.49E-04	3.36E-02	2.94E-02	S100a8/S100a9/Rock1/Pink1/Rragc/Dap/Map1lc3a/Park7/Atpif1	9
GO:0042775	mitochondrial ATP synthesis coupled electron transport	2.54E-04	3.36E-02	2.94E-02	Pink1/Cox4i1/Ndufs6/Park7	4
GO:0014812	muscle cell migration	2.74E-04	3.38E-02	2.96E-02	Rock1/Egr1/Serpine1/Ccl5/Tmsb4x	5
GO:0045931	positive regulation of mitotic cell cycle	2.84E-04	3.38E-02	2.96E-02	Numa1/Tgfa/Ccnd1/Ankrd17/Anapc11/Ddx3x	6

Table 9. Top 20 Unique Downregulated Biological Pathways in Meningeal Lymphatic Ablation Microglia IL-1β Response. Enriched downregulated biological pathways based on differentially expressed genes between ablation-saline and ablation-IL-1β microglia. Top 20 by adjusted p-value depicted. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

10.

ID	Description	pvalue	p.adjust	qvalue	geneID	Count
GO:0050663	cytokine secretion	5.51E-06	6.62E-03	4.34E-03	Nlrp3/Mbp/Notch2/Isg15/Clec4e	5
GO:0002285	lymphocyte activation involved in immune response	5.11E-05	1.48E-02	9.70E-03	Nlrp3/Notch2/Clec4d/Clec4e	4
GO:0002292	T cell differentiation involved in immune response	6.01E-05	1.48E-02	9.70E-03	Nlrp3/Clec4d/Clec4e	3
GO:0035457	cellular response to interferon-alpha	7.30E-05	1.48E-02	9.70E-03	Ifit3/Ifi204	2
GO:0002274	myeloid leukocyte activation	7.77E-05	1.48E-02	9.70E-03	Nampt/Notch2/Clec4d/Thbs1	4
GO:0042742	defense response to bacterium	8.16E-05	1.48E-02	9.70E-03	Nlrp3/Notch2/Isg15/Clec4d/Clec4e	5
GO:0038094	Fc-gamma receptor signaling pathway	8.62E-05	1.48E-02	9.70E-03	Clec4d/Clec4e	2
GO:0001819	positive regulation of cytokine production	1.02E-04	1.53E-02	1.01E-02	Nlrp3/Mbp/Clec4e/Ifi204/Thbs1	5
GO:0042110	T cell activation	1.55E-04	1.80E-02	1.18E-02	Nlrp3/Znhit1/Clec4d/Clec4e/Pag1	5
GO:0038093	Fc receptor signaling pathway	1.69E-04	1.80E-02	1.18E-02	Clec4d/Clec4e	2
GO:0002366	leukocyte activation involved in immune response	1.84E-04	1.80E-02	1.18E-02	Nlrp3/Notch2/Clec4d/Clec4e	4
GO:2000773	negative regulation of cellular senescence	1.88E-04	1.80E-02	1.18E-02	Nampt/Plk2	2
GO:0002263	cell activation involved in immune response	1.95E-04	1.80E-02	1.18E-02	Nlrp3/Notch2/Clec4d/Clec4e	4
GO:0002286	T cell activation involved in immune response	2.39E-04	2.05E-02	1.34E-02	Nlrp3/Clec4d/Clec4e	3
GO:0050714	positive regulation of protein secretion	2.86E-04	2.27E-02	1.49E-02	Nlrp3/Mbp/Adrb1/Clec4e	4
GO:0035455	response to interferon-alpha	3.03E-04	2.27E-02	1.49E-02	Ifit3/Ifi204	2
GO:0090344	negative regulation of cell aging	3.29E-04	2.32E-02	1.52E-02	Nampt/Plk2	2
GO:0002793	positive regulation of peptide secretion	4.05E-04	2.70E-02	1.77E-02	Nlrp3/Mbp/Adrb1/Clec4e	4
GO:0050715	positive regulation of cytokine secretion	5.48E-04	3.46E-02	2.27E-02	Nlrp3/Mbp/Clec4e	3
GO:0001773	myeloid dendritic cell activation	6.12E-04	3.67E-02	2.41E-02	Notch2/Clec4d	2

Table 10. Top 20 Unique Upregulated Biological Pathways in Meningeal Lymphatic Ablation Microglia IL-1β Response. Enriched upregulated biological pathways based on differentially expressed genes between ablation-saline and ablation-IL-1β microglia. Top 20 by adjusted p-value depicted. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

11.

gene_id	logFC	Pval	pct.1	pct.2
Sbno2	2.1538	0	72.41	34.14
Cebpb	2.1230	0	95.09	75.10
Cebpd	1.8204	1.03E-294	78.27	54.84
Runx1	1.3584	4.00E-277	79.87	69.48
Ccl12	2.5279	6.01E-241	81.22	49.08
Mcl1	1.2041	1.30E-224	86.68	74.34
Socs3	2.1504	5.97E-214	82.77	25.34
Bach1	1.4121	1.74E-191	67.85	47.23
Irf2bpl	1.2105	1.55E-175	66.30	43.82
Icam1	1.5050	1.18E-171	67.25	38.75
Stat3	1.2935	1.87E-168	64.50	41.36
Arid5a	2.0536	7.44E-165	48.22	10.91
Gpr84	1.7200	1.15E-164	63.95	41.83
Mt1	1.4109	2.20E-163	78.72	61.29
Zfand5	1.3937	5.14E-163	68.45	52.16
Btg1	0.9845	5.14E-163	88.63	78.83
Srgn	1.1397	7.95E-163	86.43	78.14
Ptpn1	0.9977	3.16E-156	83.43	70.39
Map3k8	1.9497	1.17E-155	47.82	10.44
Kdm6b	1.8888	2.18E-148	54.53	13.81
Etv3	1.4123	1.92E-142	52.58	30.66
Il1a	1.5723	2.65E-140	53.73	31.46
Pim1	2.1589	1.60E-138	49.77	9.39
Il4ra	1.2982	1.24E-136	62.04	38.24
Cd14	1.0511	7.20E-135	85.03	78.04

Table 11. Top 25 Differentially Expressed Genes in Aged Microglia Response to IL-1β. Differential gene expression comparing microglia of aged-IL-1β vs. aged-saline. Top 25 genes by p-value depicted with log fold change (logFC) and percent of cells with observed expression in aged-IL-1β (pct.1) and aged-saline (pct.2). F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and p-value adjustment with Benjamini-Hochberg

Chapter 3 - Discussion and Future Directions

3.1 Interleukin-1β Alters Behavior Through Indirect Mechanisms

Neuronal expression of IL-1R1 under homeostatic and developmental conditions has been repeatedly confirmed by single cell RNAseq experiments and expression of IL-1R1 has been reported to increase in spinal cord neurons in a complete Freund's adjuvant induced model of inflammatory pain (Hochgerner et al, 2018; Holló et al, 2017; Rosenberg et al, 2018). Taken together, these lines of evidence strongly suggest multiple roles for IL-1 in the CNS under both homeostatic and inflammatory conditions. Large cohort-based studies have identified several neurodevelopmental disorders with immunological abnormalities and distinct cytokine profiles, fueling interest in neuronal cytokine signaling. For example, patients with Autism Spectrum Disorder (ASD) typically have more circulating IL-17 and Th17 cells (Basheer et al, 2018; Eftekharian et al, 2018). Clinical studies investigating cytokines as biomarkers for Major Depressive Disorder have consistently identified a correlation between elevated pro-inflammatory cytokine levels and depression (Ellul et al, 2016; Mota et al, 2013). Patients with mood disorders Major Depressive Disorder and Post Traumatic Stress Disorder exhibit elevated levels of IL-1β in serum and increased IL-1β production by monocytes in response to stimulation (Gola et al, 2013; Zhang et al, 2018). These findings have established IL-1β as a biomarker for mood disorders and raised the possibility of IL-1β based therapies (Koo & Duman, 2009). The association between IL-1β and mood disorders may also have broader implications for the study of sickness behavior. Whether mood disorders can be viewed as inappropriate induction of sickness behavior is now an open question and active area of research.

Despite these indications that physiologically relevant IL-1β signaling occurs within the CNS in the context of sickness behavior and psychiatric disorders, our investigation does not

identify a meaningful contribution of IL-1β signaling in the brain with respect to generating peripheral inflammation induced behavioral change. This conclusion is based upon our findings that conditional deletion of IL-1R1 on brain endothelium, neurons, astrocytes, or microglia failed to alter the behavioral response to peripheral inflammation as assessed by locomotor activity over time, and that local production of IL-1β in the brain is not required to induce sickness behavior. Our confidence is bolstered by independent confirmation by other groups using alternative conditional deletion strategies and experimental models (Knoll et al, 2017; Liu et al, 2019). This conclusion does not preclude the possibility that IL-1β signaling within the brain impacts a facet of sickness behavior that our experimental strategy is unable to resolve or that redundant compensatory mechanisms exist. Additionally, despite the assumption that IL-1β cannot cross the blood-brain-barrier to directly access the brain parenchyma, it is possible that small amounts of IL-1β could access the brain through unknown mechanisms and still exert significant downstream effects.

The mouse models generated over the course of this investigation, in particular cell-type specific conditional knockout of IL-1R1 for CNS populations, will be useful for further interrogation of IL-1β signaling within the brain in the context of homeostatic function and models of psychiatric disease. Although we do not identify a significant role for IL-1β signaling within the brain in the context of sickness behavior, compelling evidence exists to suggest potential roles for CNS IL-1β signaling in: regulating sleep, learning and memory, behavioral conditioning, post-traumatic stress disorder, and addiction (Brambilla et al, 2010; Hutson et al, 2017; Jones et al, 2015; Jones et al, 2018; Parekh et al, 2020; Règue et al, 2019). Relatively miniscule quantities of IL-1β can elicit a powerful systemic response, therefore any homeostatic function in the brain must be tightly controlled and thus difficult to detect. As James Krueger

speculated with regard to how IL-1β could both promote sleep, which is associated with decreased body temperature, and fever, "one might assume that it is delivered by precise mechanisms, perhaps in concert with other sleep and temperature modulators." (Krueger et al, 1984). Despite multiple lines of relatively strong circumstantial evidence, a direct mechanism of IL-1β signaling on neurons in vivo has yet to be described. The mouse lines generated for this study are valuable genetic tools which enable us to assess in vivo, the contribution of IL-1β signaling in the CNS with respect to a role in modulating aspects of sleep, stress, and experimental models of psychiatric disease. Superficial preliminary phenotyping of these mouse lines has yet to reveal a cognitive phenotype however thorough interrogation of the stress response, sleep characteristics, and addictive behavior may elucidate roles for IL-1β in the brain.

3.2 Meningeal Lymphatic Vessels Facilitate Microglia Activation in Response to Peripheral Inflammation

Impaired meningeal lymphatic vessel function has been linked to decreased recirculation of CSF through the brain via the glymphatic system and worsened cognitive function in adult mice via unknown mechanisms (Da Mesquita et al, 2018b). Although there have been investigations into how meningeal lymphatic vessels contribute to Alzheimer's Disease pathology and immune surveillance of the brain in the contexts of autoimmune disease and cancer, there are many lingering questions pertaining to how this structure contributes to physiological behavioral response to stimuli, inflammatory stimuli in particular (Da Mesquita et al, 2018b; Hu et al, 2020; Louveau et al, 2018; Song et al, 2020). Prior to this investigation, we theorized that meningeal lymphatic ablation could impact sickness behavior in one of two ways. First, if paracrine signals must be released in a discrete location within the brain then perfuse toward the cellular targets, lymphatic ablation and thus glymphatic disruption may impede necessary signaling events and prevent or delay the behavioral response. Second, if the cellular targets of paracrine signals are near the source of paracrine signals, impaired clearance of solute may result in prolonged or hyperactivation of cellular targets as clearance of the paracrine signals should also be impaired. Naturally, a third possibility exists in which there is simultaneous hyperactivation and hypoactivation of different target cell populations depending on proximity to the cellular sources of paracrine signals and regional differences in glymphatic activity which is supported by our transcriptional dataset.

Here we have shown that experimental lymphatic ablation suffices to exacerbate the behavioral response to peripheral IL-1β, and that off-target effects of the lymphatic ablation drug do not contribute to this phenotype. We find that lymphatic ablation increases the magnitude of the behavioral response to peripheral inflammation however, the total duration of

the sickness behavior response remains unchanged. To interrogate how meningeal lymphatic ablation increases the severity of sickness behavior, we sequenced microglia sorted from the brains of meningeal lymphatics-ablated and sham treated mice 2 hours following i.p. injection with IL-1β or saline, the timepoint which was associated with the peak of the behavioral response. Microglia are the tissue resident immune cell of the CNS and therefore an important population to understand within the context of the CNS response to peripheral inflammation. Due to their ability to upregulate cytokines and prostaglandin synthesis in response to peripheral inflammation and recent findings indicating that they may be required to terminate sickness behavior, microglia are widely believed to play a central role in this response (Duan et al, 2018; Shemer et al, 2020).

In our analysis, we observe a profound decrease in the number of differentially expressed genes (DEGs) in meningeal lymphatics-ablated microglia in response to peripheral IL-1β relative to the sham control (1254 DEGs in sham response vs. 847 DEGs in meningeal lymphatics ablation response, **Supplemental file**). Reflected in this finding is a similar reduced phenotypic shift from homeostatic (clusters 0, 1, 3, 6) towards a response to peripheral inflammation (cluster 2) observed in meningeal lymphatics-ablated mice responding to IL-1β. These findings indicate that in the absence of functional meningeal lymphatic vessels, microglia fail to fully respond to peripheral inflammation. This activation deficit is corroborated by histological evidence demonstrating that there is a reduced morphological shift in microglia of lymphatics-ablated mice in response to IL-1β. Although this finding is seemingly contrary to contemporary theories which purport microglial production of proinflammatory factors drive sickness behavior, we assess microglia expression of proinflammatory cytokines IL-1β, IL-6, and TNFα, as well as COX-2 which is the enzyme responsible for prostaglandin E2 synthesis.

Interestingly, we discover few differences between microglia cytokine production between sham and lymphatics ablated mice in response to IL-1β. We find that IL-1β, TNFα, and COX-2 are all significantly upregulated in response to peripheral IL-1β, regardless of meningeal lymphatic ablation, while no difference in IL-6 expression is found across all groups. Two of these genes, IL-1β and TNFα, were impacted by meningeal lymphatic ablation. TNFα expression was elevated in lymphatics ablated microglia relative to sham following saline injection however no difference was detected between peripheral IL-1β treated groups. This may imply an elevated baseline level of inflammation in lymphatics-ablated mice which is also reflected in histological Sholl analysis. Conversely, IL-1β expression was equivalent among saline treated groups however in response to peripheral IL-1β, expression is significantly higher in the lymphatics-ablated group. Curiously, this is due to a higher magnitude of expression in fewer cells as IL-1β expression was identified in 32% of sham microglia but only 25% of lymphatics ablated microglia responding to peripheral IL-1β indicating that while overall microglia response is suppressed, there may be a subset of hyperactivated microglia.

We further investigate potential functional differences between the sham and lymphatics-ablated response to peripheral IL-1β by identifying enriched biological pathways in differentially expressed genes induced by peripheral IL-1β injection. In line with fewer transcriptional changes in lymphatics ablation microglia responding to peripheral IL-1β, we observe fewer enriched upregulated and downregulated biological pathways in microglia of lymphatics ablated mice. Additionally, we detect 70 unique enriched pathways in the meningeal lymphatics ablation response to IL-1β whereas we identify 381 unique enriched pathways in the sham response (**Supplemental file**). While investigating the functional nature of these uniquely enriched pathways, a trend emerges in which unique pathways to the sham

response concern energy production and demand, while the pathways unique to the meningeal lymphatics ablation response to peripheral IL-1β concern immune activation and cytokine secretion.

We interpret these findings as evidence that meningeal lymphatic ablation impairs microglia activation through suppression of paracrine signaling. Our theoretical framework supports both broad suppression of the microglia transcriptional response as well as constrained hyperactivation of a subset of microglia as indicators of aberrant paracrine signaling. To further investigate this phenomenon, a more in-depth histological analysis of microglia throughout the brain in response to IL-1β is required. Single cell RNA sequencing erases the spatial context of the cell, so we do not know the full spatial distribution of hyperactivated and hypoactivated microglia in this model. The spatial context of these cells is critical to understand two-fold. First, understanding the distribution of hyper-activated vs. hypo-activated microglia within the brain may reveal insights into novel routes of paracrine signal perfusion. Second, the spatial context of activated microglia is critical to understanding how they interact with other cell types of the brain. The next step here will be to analyze microglia morphology both by anatomical region and by proximity to actively firing neuron populations as microglia response may also be influenced by neighboring cell activity and vice versa.

Finally, despite not being the target of this investigation, the saline injected control conditions of the single-cell RNAseq data set generated may prove to be extremely valuable in their own right to assess the affects of acute meningeal lymphatic dysfunction. We identify 574 differentially expressed genes, accounting for over 1000 differentially enriched biological pathways, in meningeal lymphatics-ablated microglia versus sham microglia without IL-1β stimulation (**Supplemental file**). In line with what we report in this study, upregulated

biological pathways in meningeal lymphatics ablation tend to reflect elevated baseline activation but interestingly, the top downregulated biological pathways in meningeal lymphatics ablation microglia versus sham in the absence of IL-1β indicate aberrant circadian rhythm. Further analysis of this dataset and phenotyping of circadian rhythm deficits in meningeal lymphatic ablated mice may reveal key insights into how microglia and meningeal lymphatic function contribute to the regulation of circadian rhythm and sleep.

3.3 A Reassessment of the Role of Microglia to Sickness Behavior

Microglia are theorized to contribute to the induction and sustaining of sickness behavior through the release of soluble paracrine signals including inflammatory cytokines and prostaglandins, or through glutamate release-induced activation of N-methyl-D-aspartate (NMDA) receptors (Dantzer, 2018). Our finding that ablation of meningeal lymphatic vessels both impair microglia response and increase the severity of peripheral inflammation induced sickness behavior is seemingly in conflict with prevailing theories, particularly because we do not observe any correlation between more severe sickness behavior and elevated expression of genes responsible for soluble inflammatory paracrine signals (*Il6*, *Tnf*, *Ptgs2*) associated with sickness behavior (Matsuwaki et al, 2017). We do observe elevated *Il1b* expression in lymphatics ablated, IL-1β responding microglia however, the relevance of this is undercut by our finding that local production of IL-1β within the CNS is not required to induce sickness behavior. If microglial secretion of cytokines is, in fact, involved in producing the behavioral response to peripheral inflammation, redundant pathways must exist which can adequately compensate such that any difference in behavior was undetectable. Our speculation that microglia serve a role in tempering the severity of peripheral inflammation-induced sickness behavior is bolstered by recent reporting that resolution of sickness behavior depends upon microglial expression of the anti-inflammatory cytokine IL-10 (Shemer et al, 2020). Although we do not observe any *Il10* expression in our microglia transcriptional dataset, it is possible that this mechanism is involved at a later time point as the referenced report only examined transcription 6 hours following peripheral LPS injection while we assess 2 hours following IL-1β injection. Our finding that microglia/macrophage depletion results in an exaggerated behavioral response to peripheral IL-1β as well as independent confirmation by another group

using a peripheral LPS-induced sickness behavior model lend further support to our conclusion that microglia have a role in tempering the severity of sickness behavior (Vichaya et al, 2020).

Regardless of this speculation, it is necessary to address the competing hypothesis, that exaggerated symptoms in response to peripheral inflammation in microglia depletion models is due to astrocyte (over)compensation, if this novel role is to be verified (Vichaya et al, 2020). Additionally, further interrogation of the microglia response between 3-6 hours following peripheral IL-1β in meningeal lymphatics ablation is required to assess the potential mechanisms by which microglia may act to resolve sickness behavior including assessment of IL-10 expression as that is currently the only proposed mechanism by which microglia may suppress sickness behavior (Shemer et al, 2020). It may also provide valuable insight into any neuromodulatory role of microglia to probe further into how neuronal activity differs throughout sickness behavior in the context of microglia depletion.

In light of these findings, it may be appropriate to modify our view of microglia in sickness behavior from one of an instigator of inflammation to that of an enforcer of homeostasis. The brain is a delicate organ with reduced capacity for healing relative to other organs and as such, the tissue-resident immune cell of the brain may have evolved in such a way to tightly control homeostasis in the event of systemic inflammation while remaining primed for further response if the immunological threat breaches the brain. It may also be necessary to broaden the scope of investigations into the sources of neuromodulatory paracrine signals, including cytokines. Reporting from our lab that interferon gamma (IFNγ) signaling on neurons regulates social behavior, and that meningeal γδ T cells influence anxiety-associated behavior in mice through IL-17a signaling on neurons, indicates that the immune cell compartment of the meninges may be a critical site for the exchange of neuromodulatory signals

from the peripheral immune system to the brain, not only in the context of acute inflammatory stress but as a consistently open line of communication between the immune system and the brain, enabling risk taking behavior to be balanced with the health and level of immunological threat of the body (Alves de Lima et al, 2020; Filiano et al, 2016). Through the perspective of adaptive value, it is conceivable that evolution has selected for mechanisms that link behavior to overall health or immunological threat. This link is potentially highly complex as different immunological signals may exist to inform the brain that the body is protected and able to take risks while others may inform the brain that there is a threat and certain activities should be avoided. For example, an individual with a faulty immune system such as with severe combined immunodeficiency (SCID), may display reduced social preference, limiting the likelihood of contracting an infection through community spread (Filiano et al, 2016). Conversely, when the individual is under immunological threat, reducing risk taking behavior through IL-17a secretion by meningeal γδ T cells may enhance survival by allowing the organism to allocate more energy to rest and repair (Alves de Lima et al, 2020).

3.4 Deterioration of Meningeal Lymphatic Vessels with Age May Contribute to Age-Associated Psychiatric Pathologies

The substantial aggravation of the behavioral response to inflammation with age is well established within animal models however the cause and full medical implications remain unclear (Godbout et al, 2008; McLinden et al, 2012). Clinical data shows that elderly patients are substantially more likely to be admitted for extended hospital stays than any other age group and that extended periods of immobility are associated with increased incidence of hospital acquired disease indicating high medical value in understanding the mechanisms of sickness behavior (JW. & V., 2019; Pavon et al, 2020). Advanced age is also strongly associated with neurodegenerative disease, sleep disturbances, and increased accumulation of protein aggregates in the brain, each of which has a connection to meningeal lymphatic vasculature and glymphatic function which decline with age (Holth et al, 2019; Kress et al, 2014; Nedergaard & Goldman, 2020; Ohashi et al, 2021; Paradise et al, 2021). These relatively recent findings regarding lymphatic/glymphatic decline with age provide a new perspective by which to view old observations. For example, a study of elderly patients in 1987, in which all admitted patients were screened for emotional disorder and cognitive impairment, found that individuals with cognitive impairment required significantly longer hospital stays and incredibly, many patients who exhibited cognitive impairment before being treated were discharged without signs of cognitive impairment (Johnston et al, 1987). Taken together, these findings suggest that meningeal lymphatic deterioration may lie at an intersection between cognitive impairment, response to infection, and age-related pathology, as a critical event upstream of diverse pathologies.

Microglia hyperactivation in particular is strongly associated with advanced age in both humans and animal models, as a facet of the broader trend of an increased inflammatory profile

with age, commonly termed 'inflammaging' (Conway et al, 2017; Fonken, 2018; Streit et al, 2004; Wynne et al, 2010). This phenomenon is reflected in our own transcriptional dataset of aged mice however, it also reveals a potential contradiction regarding our adult mouse lymphatic ablation transcriptional dataset, which reveals widespread suppression of microglia activation albeit with a subset of hyperactivated microglia. I interpret this to be reflective of the short-term nature of our meningeal lymphatic ablation model, as microglia priming towards a more inflammatory state may require prolonged lymphatic dysfunction and the accumulation of other factors with age. Notably, we did observe elevated *Tnf* expression in meningeal lymphatics-ablated mice following saline injection relative to the sham control indicating some level of elevated inflammation with only one week of meningeal lymphatic ablation.

To identify commonalities between the aged response to peripheral inflammation and meningeal lymphatic ablation, we integrated the transcriptional dataset of aged mice with our dataset of adult mice, and compared differential gene expression of microglia between saline and IL-1β conditions for young (sham, 2-month-old), meningeal lymphatics-ablation (ablation, 2-month-old), and geriatric (2-year-old) mice. Similar to the suppression of the microglia transcriptional response observed in meningeal lymphatics ablation, microglia of aged mice had fewer upregulated genes than sham but more than meningeal lymphatics ablation however, the aged group also had the highest number of downregulated genes induced by peripheral IL-1β. To identify similarities in the microglia response to peripheral IL-1β between lymphatics-ablated and aged mice, and to interrogate whether any similarities were widespread or restricted to a subpopulation or microglia, we identified enriched biological pathways comparing either meningeal lymphatics ablation or aged IL-1β responding microglia to their sham counterpart on a cluster-by-cluster basis. For homeostatic microglia clusters (clusters 0, 1, 3, 6), we

observed little to modest overlap in enriched pathways relative to sham (young adult) however we find overwhelming overlap in enriched pathways relative to sham in actively responding microglia (cluster 2). In fact, over 86% of enriched biological pathways in IL-1β responding microglia in meningeal lymphatics ablation are also enriched in the aged IL-1β response relative to sham. This suggests that meningeal lymphatics ablation in young mice can serve as a suitable model for meningeal lymphatic deterioration in aged mice specifically as it relates to microglia activation in response to peripheral inflammation.

We employ AAV mediated overexpression of VEGFc to enhance meningeal lymphatic vessels and glymphatic function in aged mice and report a minor but significant amelioration of the severity of sickness behavior (Da Mesquita et al, 2018b). Despite not being able to rescue aged mice to healthy adult levels, one-month lymphatic enhancement sufficed to improve sickness behavior. Interpreted together with our finding that meningeal lymphatic ablation exacerbates sickness behavior in adult mice in response to peripheral IL-1β and that microglia activation is impaired by meningeal lymphatics ablation in a manner similar to aging, we conclude that meningeal lymphatic deterioration is a contributing factor to exaggerated peripheral cytokine induced sickness behavior with age.

These results emphasize the importance of meningeal lymphatic vessels as a therapeutic target to address age-related pathologies however, in light of the incomplete rescue of exacerbated sickness behavior by experimental enhancement of meningeal lymphatic vessels in aged mice, the efficacy of prophylactic enhancement of meningeal lymphatics throughout adulthood must be assessed to address whether inflammaging can be avoided by preventing the priming of microglia over long periods of glymphatic decline. Potentially the most tantalizing unresolved question of this investigation could be addressed by transcriptional profiling of

microglia from aged, meningeal lymphatic enhanced mice responding to peripheral IL-1β. This would be an extremely valuable addition to the datasets generated throughout this study because at present, we possess information that provides insight into how meningeal lymphatic deterioration alters microglia activation and leads to a sustained proinflammatory state however, we lack data concerning reversible affects of aging, that is whether meningeal lymphatic enhancement could boost microglia activation in response to peripheral inflammation.

3.5 Concluding Remarks - Further Evidence of the Contribution of Meningeal Lymphatic Vessels to Cognition

Sickness behavior has been described as an altered motivational state characterized by decreased overall movement, decreased interest in normal activity, impaired concentration, and lethargy (Dantzer, 2018). Despite its ubiquitous presence in the human experience, high degree of adaptive value, and extensive investigation over nearly four decades, the molecular mechanisms underlying the behavioral response to inflammation has remained elusive. Unquestionably, sickness behavior is a highly complex response, incorporating a many cell-to-cell signaling events, between numerous diverse cell populations, utilizing a broad spectrum of signaling molecules, to facilitate communication between the immune system and CNS and produce specific set of behaviors to enhance survival. In this study, we generate a simplified model of sickness behavior to enable investigation into multiple aspects of the CNS response to peripheral inflammation, including: identifying cellular targets and sources of IL-1β in the CNS, assessing the contribution of meningeal lymphatic function in facilitating the CNS response to peripheral inflammation, and characterizing the contribution of age-related meningeal lymphatic deterioration to exaggerated sickness behavior with age.

The purpose of this state is to theoretically facilitate recovery by allowing the body to conserve energy needed for an immune response however, due to conspicuous overlap in behavioral symptoms of sickness behavior, namely lethargy, anhedonia, and impaired concentration, it has been theorized that inappropriate induction of sickness behavior may contribute to the pathogenesis of psychiatric illness including major depressive disorder (Mota et al, 2013). In fact, IL-1β the initiator of sickness behavior in our experimental model, is implicated in a wide variety of psychiatric conditions where it is believed to act within the CNS.

These conditions include: Alzheimer's Disease, post-traumatic stress disorder, depression, addiction, and sleep deprivation (Alam et al, 2004; Ellul et al, 2016; Martinez et al, 2018; Spivak et al, 1997; Zumkehr et al, 2018). Lymphatic, glymphatic, and cognitive function have been extensively reported to decline with age in humans and animal models (Da Mesquita et al, 2018b; Ma et al, 2017; Paradise et al, 2021). Additionally, sleep disturbances, which disrupt glymphatic function, are associated with increased incidence of dementia underscoring the importance of the lymphatic/glymphatic system in healthy cognition (Spira et al, 2014). The elevated levels of microglial IL-1β detected in meningeal lymphatics ablated mice following peripheral IL-1β injection may imply a priming or propensity for such pathologies associated with meningeal lymphatic decline however, further investigations are required to confirm this link.

Here we demonstrate that local IL-1β production within the CNS is not required to induce a behavioral response to inflammation nor is IL-1β signaling on neurons, brain endothelial cells, astrocytes, or microglia. Each of these cell populations possess the capacity to respond to IL-1β as indicated by transcription of the necessary receptor, generating speculation that they may be involved in sickness behavior, however, our data and independent confirmation by another group do not indicate a significant role for IL-1β on these CNS cell populations in sickness behavior (Liu et al, 2019; Rosenberg et al, 2018). We further evaluate the role of microglia by assessing sickness behavior under microglia depleted conditions and unexpectedly observe significantly more severe sickness behavior in the absence of microglia and tissue resident macrophages. Although this finding was unanticipated due to enduring speculation that microglia drive sickness behavior through secretion of inflammatory factors, this finding has since been independently confirmed in a different model of peripheral inflammation induced

sickness behavior and a potential role for microglia in terminating sickness behavior has recently been described (Shemer et al, 2020; Vichaya et al, 2020). The reassessment of the role of microglia as a tempering or moderating force in sickness behavior is further bolstered by our finding that severe sickness behavior in our experimental meningeal lymphatic ablation model is associated with a suppressed and deficient microglia response. Finally, we show that experimental meningeal lymphatic enhancement in aged mice is sufficient to reduce the severity of sickness behavior, underscoring the importance of healthy lymphatic/glymphatic function in orchestrating an appropriate behavioral response to inflammation.

Prior to the work reported here, microglia activation and subsequent release of inflammatory signal molecules, including IL-1β, was purported to be the driving force responsible for producing a behavioral shift in response to peripheral inflammation and glymphatic function was not recognized as contributing force in sickness behavior (Dantzer, 2018). Although our finding that microglia and macrophage depletion results in more severe sickness behavior in response to peripheral inflammation has been independently confirmed, the paradigm of microglia/neuroinflammation driven sickness behavior persists due to the possibility that either the other neural cell populations or the remaining ~1% microglia could compensate for the broad depletion of microglia (Vichaya et al, 2020). Here we demonstrate a clear contribution of meningeal lymphatic function to the behavioral response to peripheral inflammation with clinical relevance to age related pathology. Further interrogation of the transcriptional dataset generated may reveal additional insights into meningeal lymphatic control over circadian rhythms in microglia and impacts on cognition. Moreover, we find that IL-1β signaling on key immune cell populations is dispensable to induce a behavioral response to peripheral inflammation and in fact, IL-1β production within the brain is entirely unnecessary

to induce sickness behavior. Transcriptional profiling of microglia from meningeal lymphatics ablated mice reinforce this concept and shed new light on the contribution of meningeal lymphatic function to microglia response, as impaired activation is associated with more severe behavioral output. I believe that a thorough reevaluation of the role of microglia in the context of these new findings is an essential prerequisite to properly understand this phenomenon, uncover links to psychiatric disorders, develop new therapeutic treatments for psychiatric illness, and combat cognitive decline with age.

Chapter 4 – Acknowledgements

The preceding thesis and completion of my PhD training would not have been possible without the substantial contributions, support, and mentorship of many individuals. Above all, I must thank my graduate mentor Dr. Jonathan Kipnis whose tutelage, and personal generosity cannot be overstated. When I entered the Biomedical Sciences Graduate Program, I was not a neuroscientist or an immunologist, nor did I have particular aspirations to be either, however, as I rotated through various labs, I was struck by the incredible scientific environment that Dr. Kipnis had cultivated by bringing together diverse areas of expertise to investigate bold new concepts and identified it as an ideal learning environment. The level of both personal and professional support that I received from Dr. Kipnis throughout my graduate education goes beyond any I have experienced outside of my family tree. Regardless of any disagreements or past tensions with my mentor, one thing that cannot be denied is Dr. Kipnis' dedication to ensuring the success of each of his students and trainees. I can say without hesitation that being a member of the Kipnis Lab has been the greatest opportunity of my life and I will forever be grateful for the privilege.

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benchwork throughout my time in the Kipnis Lab has been critical to my education. During my tenure in the Kipnis Lab, I have been privileged to work with incredible postdoctoral fellows including, Dr. Anthony Filiano, Dr. Sandro Da Mesquita, and Dr. Antoine Louveau, each of whom has since moved on to lead their own lab. Each of these scientists has been instrumental in my in my education and the impact of their guidance can be seen embedded throughout this thesis. I am also indebted to the people who ensure that the lab runs smoothly. The surgical expertise of veterinarian Dr. Igor Smirnov dramatically expands the experimental and learning opportunities open to lab members and allows us to push the boundaries of our field. In my time as a graduate student, the size of the Kipnis Lab has more than doubled and we owe our ability to maintain a rigorous pace of research to the dedicated support staff including, lab managers Wendy Baker and Sean Brophy, animal technician Nour Al Hamadani, and all other support staff both within the Kipnis Lab and at the University of Virginia and Washington University who maintain our mouse colonies and ensure that we have the equipment we need to pursue our investigations.

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Chapter 5 – Materials and Methods

Mouse models and tissue collection

All mice used were on C57BL/6 background and bred in-house using mice purchased from The Jackson Laboratory. Mice were maintained under specific pathogen-free conditions, with controlled temperature and humidity, on 12 h light:dark cycles, and ad libitum access to food and water. For conditional knockout experiments *IL1R1*$^{flox/flox}$ (Jax:028398) mice were crossed with *Syn1*^{Cre} (Jax:003966), *Cx3cr1*^{CreERT2} (Jax:020940), or *GFAP*^{CreERT2} (Jax:012849) strains, or administered AAV as described in 'AAV delivery' to induce conditional deletion of *IL1R1* on brain endothelium. Cre negative littermates were used as control mice for respective experiments. *IL1B* KO mice were ordered from The Jackson Laboratory (Jax:032998) and allowed 2 weeks habituation before use in experiments. All experiments were performed with adult mice between 2-4 months of age or aged mice over 2 years of age. Aged mice were provided by the National Institutes of Health/National Institute on Aging. All experiments were approved by the Institutional Animal Care and Use Committees of the University of Virginia and Washington University. For all tissue collection, mice administered a lethal dose of anesthetic (Euthasol) and transcardially perfused with cold PBS.

Tamoxifen Treatment

Tamoxifen inducible Cre mouse lines, including Cre negative littermates were provided Tamoxifen chow (ENVIGO) for 2 weeks to induce conditional excision of floxed alleles. Tamoxifen chow was then replaced with normal chow for 2 weeks prior to testing to reduce off-target affects of Tamoxifen treatment.

IL-1β Induced Sickness Behavior

Recombinant murine IL-1β was obtained from Peprotech and resuspended in sterile saline. Mice were administered 1μg IL-1β or saline vehicle in 100μL i.p. injection for all sequencing and adult mouse behavior. Due to poor tolerance of IL-1β in aged mice, the dose was reduced to 0.5μg for behavior experiments.

Immunohistochemistry, imaging and quantification

Mouse brain and skull cap were drop fixed in 4% paraformaldehyde at 4ºC for 24 hours. Following fixation, dura was dissected from skull cap and stored in PBS containing Azide (0.02%) until further processing. Brain tissue was dehydrated in 30% sucrose solution then frozen in optimum cutting temperature compound (Sakura Finetek), 60-μM sections were cut on a cryostat (Leica). Floating sections were stored in PBS containing Azide (0.02%) until further processing. Tissue samples were permeabilized in PBS containing 0.2% Tween and 0.1% Triton X-100 and blocked with 2% chicken serum in PBS containing 0.1% Tween and 0.05% Triton X-100 for 1 hour. Samples were incubated overnight at 4ºC with primary antibodies in PBS containing 2% chicken serum, 0.1% Tween, and 0.05% Triton X-100. Primary antibodies include: goat anti-Iba1 (Abcam), rat anti-Lyve1-eFluor660 (eBioscience™), and hamster anti-CD31 (Millipore). Following primary antibody incubation, tissue was washed three times in PBS containing 0.1% Tween and 0.05% Triton X-100 then incubated in fluorescently conjugated secondary antibody for 2 hours at room temperature. Tissue was then washed 3 times and incubated in DAPI for 10 minutes. Tissue was then mounted on microscope slides using Aqua-Mount (Thermo Scientific) or Prolong Gold (Invitrogen). Brain tissue was imaged using Olympus FV1200 Laser Scanning Confocal

Microscope and meninges was imaged using Olympus VS200. All image quantification was performed using the FIJI package for ImageJ. Sholl analysis was performed by manually isolating individual microglia and using the Sholl analysis plugin for ImageJ. Lymphatic vessel diameter was quantified by manually annotating lymphatic vessel diameter on at least 100 points along the superior sagittal sinus-adjacent lymphatic vessels. All analysis was performed blinded to conditions and the experimenter was unblinded following statistical analysis.

Behavior analysis

All behavior tests were performed during light cycle and mice were habituated to the testing room in home cage for at least one hour prior to behavioral testing. For repeated open field test, mice were placed in the corner of a 35cm x 35cm arena and recorded for 10 minutes then returned to their home cage. Mice are tested four times, at 1, 2, 3, 6, and 24 hours following IL-1β or vehicle treatment. Following each open field test, mice are returned to their home cage and remain in the testing room until all time points have been collected. All behavioral testing was recorded on video. Mouse tracking and analysis was performed using EthoVision (Noldus) software to quantify locomotor activity. Immobility was detected by behavioral software when the subject velocity dropped below 1.75 cm/s and movement resumed when subject velocity rose above 2 cm/s. Accuracy of detection settings was confirmed by visual summary. Statistical outliers were identified using ROUT (Q=0.5%) and excluded from analysis. Analysis and graph plotting were performed using Prism 9 (GraphPad). All analysis was performed by an experimenter blinded to experimental groups.

Meningeal lymphatic ablation

Visudyne (verteporfin, Valeant Ophtalmics) solution used for lymphatic ablation was reconstituted according to manufacturer instructions. Anesthetized mice were administered 5µL of Visudyne solution via intra-cisterna magna injection and placed on a heating pad for 15 minutes to allow the drug to collect in lymphatic vessels. The drug was then photoconverted through intact skull using a non-thermal 689-nm wavelength laser (Coherent Opal Photoactivator, Lumenis) at 5 points previously demonstrated to be effective (Da Mesquita et al, 2018b). Each point was photoconverted for 83 seconds to provide a light dose of 50 J/cm^2 at an intensity of 600 mW/cm^2. Following photoconversion, the skin was sutured, post-surgery analgesic and antibiotic were administered, and mice were allowed to recover on heating pads until fully awake. For sham procedure, mice either received artificial CSF instead of Visudyne or were not photoconverted following Visudyne injection. Behavior experiment results were replicated using both sham treatments in separate experiments. For microglia sequencing, artificial CSF control was used.

Intra-cisterna magna injections

Intra-cisterna magna injections were performed as previously described (Da Mesquita et al, 2018b). Injections were performed on mice secured in a stereotaxic frame, under ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) anesthesia. The incision area on the back of the neck was prepped for surgery by shaving and iodine antiseptic and ophthalmic ointment was applied to the eyes. Upon making an incision, the muscle layers above the cisterna magna were retracted and the solution was injected using a Hamilton syringe with 33-gauge needle. After removing the needle and suturing the skin, mice were administered analgesic (2 mg kg^{-1}

ketoprofen) and antibiotic (2.5 mg kg^{-1} Baytril) solutions and allowed to recover on heating pads until fully awake.

AAV delivery

All AAV vectors used were purchased from Vector Biolabs. For brain endothelium conditional knockout experiments, AAV-BR1-CAG-Cre or AAV-BR1-CAG-gGFP were administered by $5x10^{11}$ GC per mouse by vein injection and allowed one week recovery for vector expression as previously described (Körbelin et al, 2016). For lymphatic enhancement experiments in aged mice, 10^{13} GC of AAV1-CMV-VEGFc or AAV1-CMV-LacZ was delivered i.c.m. in 2µL of artificial CSF as described in intra-cisterna magna injections. Following i.c.m. virus injection, mice were allotted one-month recovery to allow expression and lymphatic enhancement before behavioral assessment.

Microglia depletion

The normal chow of adult mice was replaced with feed containing PLX5622 for two weeks to deplete microglia while experimental controls were placed on identical feed formulation except lacking the drug PLX5622 as previously described (Cronk et al, 2018). Depletion was verified by immunohistochemistry for Iba1.

ELISA

Following lethal dose of anesthetic, blood was collected from the retinal artery after removing the eye and placed in heparinized tubes, pooling two mice per replicate. ELISA was performed

on plasma using Invitrogen™ IL-1 beta Mouse ELISA Kit per manufacturer's instructions. Statistical analysis and graph plotting were performed using Prism 9 (GraphPad)

Microglia isolation and single cell sequencing

Mice were given a lethal dose of anesthetic and perfused with ice cold PBS with heparin. Immediately following perfusion, whole brain was harvested and divided by hemisphere for dual analysis pipelines. One hemisphere from each brain was immediately drop fixed for histological analysis while the other hemisphere was suspended in ice cold PBS for microglia isolation. At this point, hemispheres from biological replicates are pooled, five mice per condition. Following tissue collection, PBS was decanted, and tissue was dissociated in 5mL Hank's buffering saline solution with DNaseI (50U/mL) and papain (4U/mL) at 37ºC for 45 minutes. Cell suspension was then passed through a 70um strainer and CD11b+ cells were magnetically sorted using CD11b MicroBeads, human and mouse (Miltenyi) as per manufacturer's instructions using AutoMACS (Miltenyi). Enriched microglia samples were then submitted to the University of Virginia Genome Analysis and Technology Core for single cell library preparation and sequencing targeting 2000 cells per sample at 100,000 reads per cell.

scRNAseq analysis

Base call files were converted to Cellranger compatible fastq files using the Illumina Bcl2fastq software. Reads were then aligned to the mm10 transcriptome using the Cellranger software pipeline (version 2.2.0) provided by 10x genomics, specifically the count function with expected counts of 2000 cells per sample. The resulting filtered gene by cell matrices of UMI

counts for each sample were read into R using the *read10xCounts* function from the Droplet Utils package and young and old samples were merged separately by gene symbol into two dataframes (Lun et al, 2019). Further filtering was applied in order to exclude genes expressed in 5 or fewer cells and remove low quality cells. In the young samples, cells were excluded if they had fewer than 1,000 or greater than 50,000 UMI counts, cells expressing fewer than 500 unique genes, as well as cells with greater than 10% mitochondrial gene expression. In the aged samples, cells were excluded if they had fewer than 500 or greater than 35,000 UMI counts, cells expressing fewer than 500 or greater than 6,000 unique genes, as well as cells with greater than 15% mitochondrial gene expression. Expression values for the remaining cells were then normalized using the scran and scater packages and the resulting log2 values were transformed to the natural log scale for compatibility with the Seurat (v3.1.1) pipeline. Aged and young samples were then integrated with the use of *FindIntegrationAnchors* and *IntegrateData* from the Seurat package. Expression values were scaled across each gene, the effects of sequencing depth per cell and number of unique features were regressed out, and principal components analysis was applied. Based on the statistical significance of the principal components, calculated with the jackstraw test, and the percentage of variance explained by each, the first 10 components were chosen for t-Stochastic Neighbor Embedding (tSNE) and Shared Nearest Neighbor clustering. Optimization of the Louvain algorithm applied to the shared nearest neighbor graph as implemented in the *FindClusters* function with resolution set to 0.2 resulted in 11 clusters which were annotated manually based on canonical gene markers. For analysis of differentially expressed genes between conditions, each cluster was filtered to include genes that had at least 5 transcripts in at least 5 cells, then the top 2,000 highly variable genes were determined and included for further analysis using the SingleCellExperiment *modelGeneVar*

and *getTopHVG* functions. After filtering, observational weights for each gene were calculated using the ZINB-WaVE *zinbFit* and *zinbwave* functions (Van den Berge et al, 2018). These were then included in the edgeR model, which was created with the *glmFit* function, by using the *glmWeightedF* function (Robinson et al, 2010). Results were then filtered using Benjamini-Hochberg adjusted p-value threshold of less than 0.05 as statistically significant. Volcano plots were made with the EnhancedVolcano package (Blighe et al, 2020). Upset plots were made with the UpSetR package (Conway et al, 2017). Over representation enrichment analysis with Fisher's Exact test was used to determine significantly enriched Gene Ontology terms (adj. $p < 0.05$) for the sets of significantly differentially expressed genes between conditions. For each gene set, genes were separated into up- and down-regulated and separately (Hong et al, 2014) the *enrichGO* function from the clusterProfiler package was used with a gene set size between 10 and 500 genes and p-values adjusted using the Benjamini-Hochberg correction (Yu et al, 2012).

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