NEURO-IMMUNE INTERACTIONS IN INFLAMMATION AND AUTOIMMUNITY

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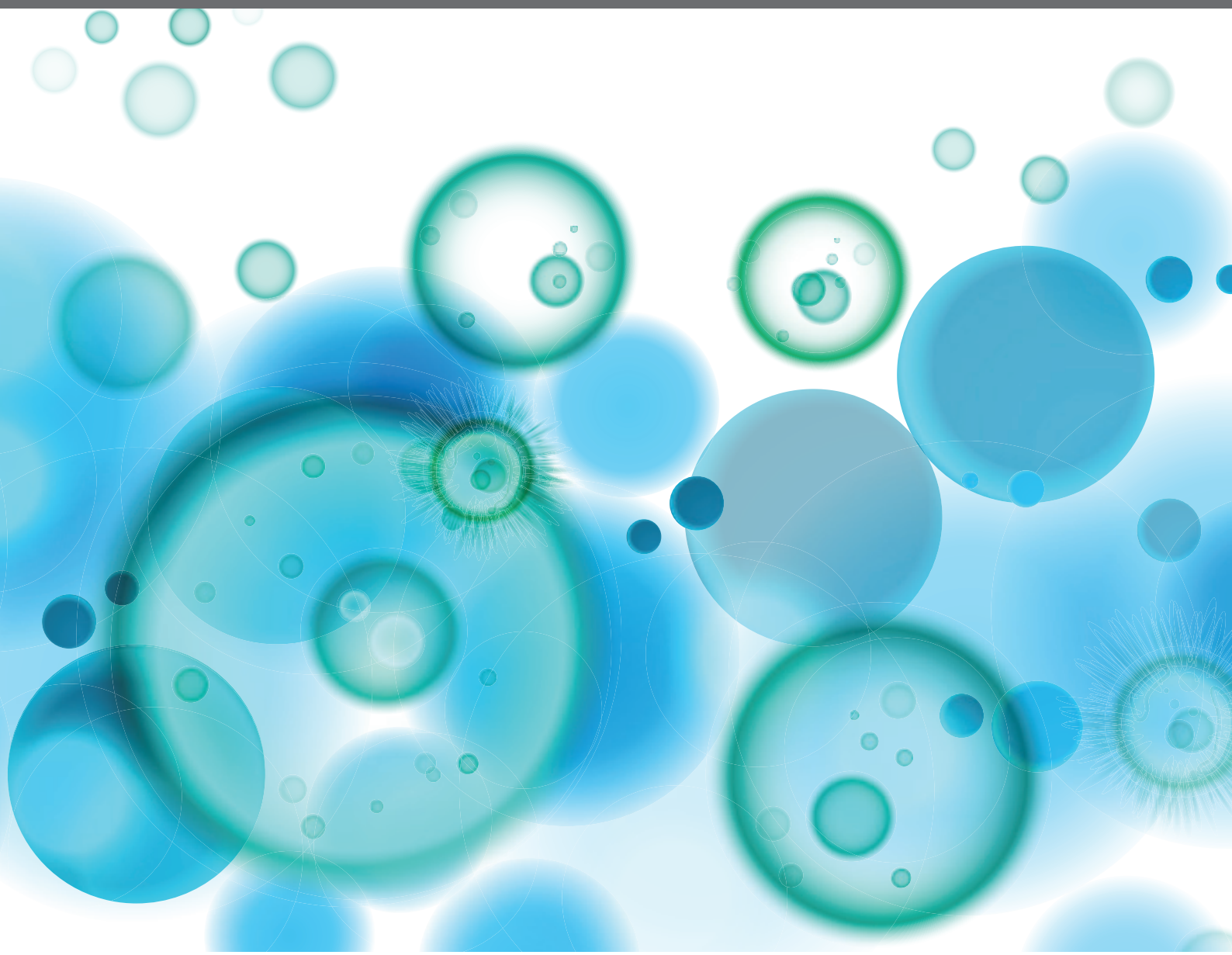

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NEURO-IMMUNE INTERACTIONS IN INFLAMMATION AND AUTOIMMUNITY

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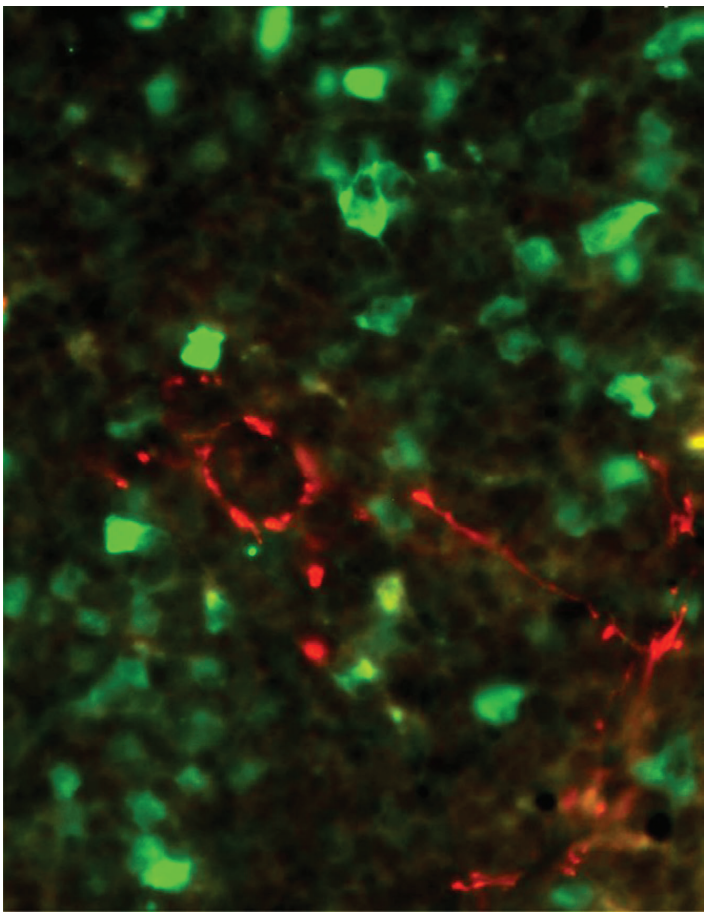

Acetylcholine-synthesizing T cells (green) in spleen are located in the proximity of catecholaminergic nerve endings (red). From Rosas-Ballina, 2011. Reprinted with permission from AAAS.
Rosas-Ballina M, Olofsson PS, Ochani M, Valdés-Ferrer SI, Levine YA, Reardon C, Tusche MW, Pavlov VA, Andersson U, Chavan S, Mak TW, Tracey KJ (2011) Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science.* 2011 Oct 7;334(6052):98-101. doi: 10.1126/science.1209985. Epub 2011 Sep 15.

The nervous system plays an important role in the regulation of immunity and inflammation. However, unbalanced immune responses in inflammatory and autoimmune conditions can exert deleterious effects on neuronal integrity and brain function. Recent studies have characterized neural pathways communicating peripheral inflammatory molecules to the CNS, and brain- and spinal cord-derived circuitries controlling various innate and adaptive immune responses and inflammation. Ongoing research has revealed cellular and molecular mechanisms underlying these neural circuits and indicated new therapeutic approaches in inflammatory and autoimmune

disorders. Pharmacological and bioelectronic modulation of neural circuitry has been successfully explored in preclinical settings of sepsis, arthritis, inflammatory bowel disease, obesity-driven disorders, diabetes and other diseases. These studies paved the way to successful clinical trials with bioelectronic neuronal modulation in rheumatoid arthritis and inflammatory bowel disease.

Dysregulated release of cytokines and other inflammatory molecules may also have severe consequences on brain function. Brain inflammation (neuroinflammation), imbalances in brain neuronal integrity and neurotransmitter systems, and cognitive dysfunction are characteristic features of post-operative complications, sepsis, liver diseases, diabetes and other disorders characterized by immune and metabolic dysregulation. Derangements in cytokine release also play a pivotal role in depression. Characteristic brain reactive antibodies in autoimmune conditions, including systemic lupus erythematosus and neuromyelitis optica, significantly contribute to brain pathology and cognitive impairment. These studies, and the simultaneous characterization of neuro-protective cytokines, identified new therapeutic approaches for treating neurological complications in inflammatory and autoimmune disorders. This Frontiers Research Topic is a forum for publishing research findings and methodological and conceptual advances at the intersection of immunology and neuroscience. We hope that presenting new insight into bi-directional neuro-immune communication in inflammation and autoimmunity will foster further collaborations and facilitate the development of new efficient therapeutic strategies.

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EDITORIAL
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Editorial: Neuro-Immune Interactions in Inflammation and Autoimmunity

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Keywords: neuro-immune interactions, immunity, inflammation, autoimmunity, cytokines, antibodies

Editorial on the Research Topic

Neuro-Immune Interactions in Inflammation and Autoimmunity

The nervous system plays an important role in the regulation of immunity and inflammation (1, 2). However, neuronal integrity and brain function may be severely altered in inflammatory and autoimmune conditions (3–5). Recent studies have characterized neural pathways communicating peripheral inflammatory signals to the central nervous system (CNS), and brain- and spinal cord-derived circuitries controlling various innate and adaptive immune responses and inflammation (1, 2). A prototypical neural reflex circuit that regulates immunity and inflammation is the vagus nerve-based "inflammatory reflex" (6). Pharmacological and bioelectronic modulation of neural cholinergic circuitry in the inflammatory reflex has been successfully explored in preclinical settings of sepsis, arthritis, inflammatory bowel disease, obesity-driven disorders, diabetes, and other diseases (1). These studies paved the way to successful clinical trials in inflammatory bowel disease, rheumatoid arthritis, and metabolic syndrome (7–9).

Dysregulated release of cytokines and other inflammatory molecules may have a severe impact on brain function (10). Brain inflammation (neuroinflammation), imbalances in brain neuronal integrity, neurotransmitter release, and cognitive impairment are characteristic features of perioperative neurological disorders, sepsis, pain, liver diseases, diabetes, and other conditions characterized by immune and metabolic dysregulation (3, 11, 12). These findings and the characterization of brain-reactive antibodies and neuroprotective cytokines indicated new therapeutic approaches for treating inflammatory and autoimmune disorders and their neurological complications (4, 13, 14).

The collection of 19 papers on this research topic substantially contributes to improved understanding of neuro-immune communication and its therapeutic relevance. Yuki et al. elaborate on neuro-immune interactions within the gateway reflexes that regulate the entry of pathogenic $CD4^+$ T lymphocytes in the CNS and neuroinflammation. They also point to the need of further characterization of the functional anatomy of these reflexes that will be of interest for their therapeutic exploration to alleviate local neuroinflammation in pathological conditions, including multiple sclerosis. Bonaz et al. review the role of the vagus nerve as a regulator of inflammatory processes in the gastrointestinal tract. They also elaborate on accumulating experimental evidence indicating possibilities to use electrical vagus nerve stimulation for therapeutic benefit in inflammatory bowel disease and other gastrointestinal disorders in preclinical and clinical settings. Innoe et al. outline new findings related to the neural vagus nerve control of inflammation with a specific focus on the kidney disease. They also summarize evidence that this neural regulation can be activated by the use of ultrasound and other modalities to alleviate inflammation and acute kidney injury in murine models. The splenic noradrenergic innervations are implicated in neuroimmunomodulation and are an important component of the inflammatory reflex through their functional cooperation with the vagus nerve. Hoover et al. provide insight into the anatomy of noradrenergic neurons in relation to leukocytes in the human spleen and experimental evidence for a significant splenic noradrenergic neuronal loss

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in patients who died from sepsis. These findings are of interest for further studies on the neural regulation of inflammatory processes in the spleen in the context of sepsis and other conditions. Fujii et al. present a thorough review of the cholinergic system, including choline acetyltransferase, acetylcholinesterase, acetylcholine, and muscarinic and nicotinic acetylcholine receptors in immune cells, such as macrophages, dendritic cells, and T and B lymphocytes. They also summarize findings about the functional role of this non-neuronal cholinergic system in the regulation of innate and adaptive immune responses. Valdés-Ferrer et al. evaluated the role of pyridostigmine, an acetyl-cholinesterase inhibitor, in a 16-week proof-of-concept open-label trial in HIV-infected patients with discordant immune responses. By using this strategy, the authors harnessed the cholinergic anti-inflammatory pathway and demonstrated that pyridostigmine can promote recovery of $CD4^+$ T-cell counts by reducing T cell overactivation. Bosmans et al. discuss the role of cholinergic signaling, mediated through nicotinic and muscarinic receptors in the regulation of allergic inflammation, including barrier function, innate and adaptive immune responses, and effector cells responses. They also point to possibilities of exploring cholinergic regulation of type 2 immune responses in the treatment of allergic diseases.

Mader et al. outline how antibodies associated with autoimmune diseases lead to brain pathology and irreversible damage, specifically focusing on conditions such as systemic lupus erythematosus and neuromyelitis optica. They summarize some of the key mechanisms involved in these antibody-mediated pathologies and discuss the urgency to develop more appropriate and less invasive treatments for antibody-mediated diseases. Foster et al. outline the latest insights into the molecular mechanisms of the relationship between sensory neurons and immune cells, which can be protective, but also maladaptive. They elaborate on the role of nociceptors (noxious stimulus detecting sensory neurons) in the regulation of adaptive immunity with a specific focus on the involvement of nociceptors in the regulation of type 2 adaptive immune responses in airway inflammation. Gunasekaran et al. report on their discovery that peripheral sensory neurons of the dorsal root ganglia (DRG) of immunized mice contain antigen-specific antibodies. The authors use a combination of molecular genetic analyses, transgenic mice, and adoptive transfer experiments, to reveal that DRGs do not synthesize antigen-specific antibodies, but sequester primarily IgG1 subtype antibodies released by antibody-secreting plasma cells. In the broader context of the role of DRG sensory neurons in regulating antigen trafficking during immunization, these findings suggest that the nervous system may cooperate with the immune system to regulate antigen-mediated responses. Maurer and Williams describe the role of cholinergic signaling in cognition from the perspective of cholinergic modulation of microglia and astrocyte function, and the role of this regulation in Alzheimer's disease and Parkinson's disease. The colleagues focus on the cholinergic modulation of hippocampus-related neuroplasticity and memory processes and the involvement of the α7 nicotinic acetylcholine receptor in neuro-immune regulation in this context. Zaghloul et al. present new data about forebrain cholinergic dysfunction in parallel with neuroinflammation and peripheral immune and metabolic dysregulation in mice surviving cecal ligation and puncture-induced sepsis. These findings suggest that brain cholinergic signaling can be further explored as a therapeutic target in chronic sepsis illness, including cognitive impairment, functional disabilities, and alarmingly high mortality. Feng et al. demonstrate that preoperative exercise prevents the cognitive decline and neuroinflammation and increases the diversity of the gut microbiome in a model of tibia fracture with internal fixation surgery in rats with metabolic syndrome. These findings are of interest for considering exercising, acting as a microbiome modulator, in alleviating persistent cognitive impairment and neuroinflammation in postoperative conditions in high risk settings. Yang et al. used a laparotomy model in mice to report on the role of the blood–brain barrier in postoperative cognitive dysfunction. They found that anesthesia/surgery triggers endothelial dysfunction in an interleukin-6-dependent and age-associated manner, with significant cognitive impairments evident in 18-, but not 9-month-old mice. These data offer additional insights into the pathogenesis of postoperative complications like delirium and postoperative cognitive dysfunction. Berger et al. report results from a clinical study designed to evaluate neuroinflammation in patients with intracranial surgery receiving anesthetic maintenance with propofol or isoflurane. They found significant increases in cerebrospinal fluid (CSF) cytokine levels 24 h after surgery (as compared with levels before surgery), in parallel with increased CSF levels of tau, a neural injury biomarker, but no significant differences in terms of the anesthetic used. These results suggest that postoperative neuroinflammation in two anesthesia regimens may have a role in neural injury following intracranial surgery. Zhang et al. describe the neuroprotective effects of an annexin A1-derived small peptide on postoperative inflammation and cognitive function after cardiac surgery in rats. They report significant attenuation of microglial activation, cell death markers, and NF-κB activation after cardiopulmonary bypass with deep hypothermic circulatory arrest and, *in vitro*, using oxygen-glucose deprivation. These results provide evidence for the use of novel resolution agonists to treat inflammatory conditions in the perioperative space. Whittington et al. review the role of inflammation and specialized pro-resolving lipid mediators in neurological conditions and Alzheimer's disease. Resolution of inflammation is well appreciated as an active process regulated by specialized pro-resolving lipid mediators derived from omega-3 fatty acids. The authors discuss on both preclinical and clinical evidence supporting the hypothesis that Alzheimer's disease is a neurodegenerative disorder where failed resolution contributes to the disease process. Huh et al. outline the role of bone marrow stem cells or bone marrow stromal cells (BMSCs) in modulating chronic pain by acting in a paracrine fashion on peripheral and CNS sites to regulate inflammation. The authors review the mechanisms of substantial anti-nociceptive efficacy of BMSCs in preclinical and clinical settings and suggest possibilities for their use in the management of chronic pain. Pittaluga reviews the brain functional cross-talk between CCL5 (RANTES), released from glial cells and glutamate, the main excitatory neurotransmitter in the brain, in physiological and pathological conditions. She focuses on preclinical data demonstrating the role of CCL5 as a modulator of glutamatergic transmission in health and in demyelinating diseases with relevance to the onset

of psychiatric symptoms and the progression of inflammation and demyelination.

Overall, the content of this research topic reflects the significant growth and advances in the field of neuro-immune interactions. It contributes to improved mechanistic understanding, which is interrelated with evaluating both pharmacological and bioelectronic approaches to modulate neural circuitry for therapeutic benefit preclinically and in human clinical trials. Further characterization of neural circuits and signaling mechanisms will be instrumental to inform the development of safer and more efficient therapeutic approaches for diseases characterized by dysregulated immune responses, autoimmunity, and inflammation.

AUTHOR CONTRIBUTIONS

Both authors wrote and approved the manuscript.

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REVIEW
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The Gateway Reflex, a Novel Neuro-Immune Interaction for the Regulation of Regional Vessels

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The gateway reflex is a new phenomenon that explains how immune cells bypass the blood–brain barrier to infiltrate the central nervous system (CNS) and trigger neuroinflammation. To date, four examples of gateway reflexes have been discovered, each described by the stimulus that evokes the reflex. Gravity, electricity, pain, and stress have all been found to create gateways at specific regions of the CNS. The gateway reflex, the most recently discovered of the four, has also been shown to upset the homeostasis of organs in the periphery through its action on the CNS. These reflexes provide novel therapeutic targets for the control of local neuroinflammation and organ function. Each gateway reflex is activated by different neural activations and induces inflmammation at different regions in the CNS. Therefore, it is theoretically possible to manipulate each independently, providing a novel therapeutic strategy to control local neuroinflammation and peripheral organ homeostasis.

Keywords: gateway reflex, inflammation amplifier, central nervous system, chemokines, pathogenic CD4$^+$ T cells, blood-brain barrier

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INTRODUCTION

The nervous system can sense various environmental stimulations such as light, sound, and temperature through the activation of specific neurons. In addition, events in social interactions that cause psychological alterations such as anxiety, depression, or euphoria can be regarded as environmental stimulations. These stimuli can cause chronic stress that is detrimental to health. A well-defined mechanism is the release of corticosteroid hormones *via* the hypothalamus–pituitary–adrenal grand axis, which systemically modulates immune responses (1, 2). In addition to systemic regulation, there exist local regulations of the inflammatory status by specific neural activations. For example, sensory neural stimulation in the soleus muscles by gravity induces chemokine expressions in the dorsal vessels of the fifth lumbar (L5) but not at other levels of the spinal cord *via* sympathetic (adrenergic) nerve activation (3). In the case of an animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), chemokine upregulation triggers the infiltration of central nervous system (CNS)-autoreactive CD4$^+$ T cells (pathogenic CD4$^+$ T cells) from the L5 dorsal vessels into the CNS (3). This unexpected neuro-immune interaction led us to hypothesize that other specific neural stimulations may locally affect immune reactions and organ functions in different ways. Local neuro-immune communications can have pro-inflammatory (3, 4) and anti-inflammatory effects (5–10). In this review, we summarize specific neuro-immune interactions that regulate neuroinflammation and organ homeostasis.

THE INFLAMMATORY REFLEX

Because clinical studies indicated that nicotine administration or smoking can improve colon inflammation, Dr. Kevin Tracey and his colleagues hypothesized that the parasympathetic (cholinergic) nervous system may regulate an inflammatory response (6, 11–13). Using a murine model of sepsis, they demonstrated that activation of the vagus nerves, which mainly consist of parasympathetic nerves, inhibits systemic inflammation. They termed this neural reflex as the "inflammatory reflex" (14–18). In this example of the inflammatory reflex, lipopolysaccharide (LPS) injection in mice induced norepinephrine (NE) secretion in the spleen through the activation of splenic and vagus nerves. They found a novel subset of $CD4^+$ T cells that produce acetylcholine in response to NE. The released acetylcholine was found to act on macrophages that express α7 nicotinic receptor and suppress the LPS-induced inflammatory response (**Figure 1**) (14). This cascade also promotes anti-inflammatory reactions during ischemia–reperfusion injury (5, 7). A more recent study revealed that direct stimulation of C1 neurons in the medullary reticular formation of the brain conferred the same anti-inflammatory effect (5). It is also reported that stimulating mice by electroacupuncture at the Zusanli acupoint (ST36) located near the common peroneal and tibial branches of the sciatic nerve or by direct stimulation of the sciatic nerve inhibits septic shock through vagal activation and dopamine production (9). Furthermore, local ultrasound also induces the anti-inflammatory splenic neuro-immune interaction (19). Recently, the inflammatory reflex has been tested in a first-in-human trial with promising results (20). These findings solidify a scientific basis for acupuncture and physical therapy.

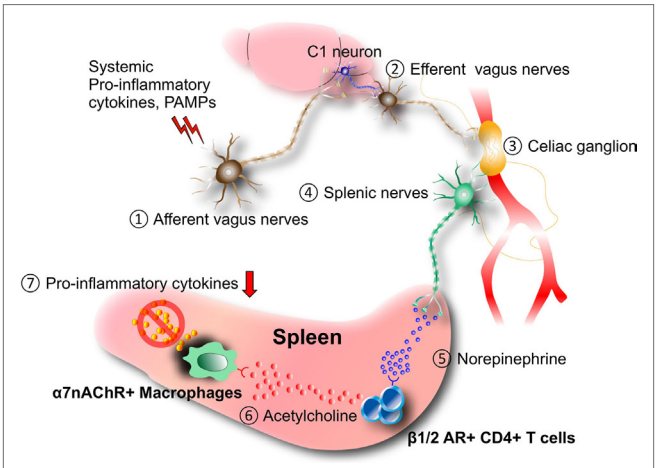

FIGURE 1 | Inflammatory reflex. Afferent (1) and efferent vagus nerve (2) stimulation by systemic pro-inflammatory cytokines and/or pathogen-associated molecular patterns (PAMPs) during septic shock, ischemia–reperfusion injury, and other inflammatory conditions induces neural activation in the celiac ganglion (3), followed by the production of norepinephrine (NE) from the splenic nerves (4). NE (5) stimulates the release of acetylcholine from a novel $CD4^+$ T cell subset that expresses choline acetyltransferase and β1/2 adrenergic receptor (AR) (6). Released acetylcholine (6) acts on macrophages expressing α7 nicotinic acetylcholine receptor (α7nAChR) to inhibit the expression of pro-inflammatory cytokines including TNFα. It is suggested that C1 neurons in the medullary reticular formation of the brain mediate the anti-inflammatory effect.

THE GATEWAY REFLEXES

Gravity-Gateway Reflex

Since the CNS historically has been considered as an immunologically privileged site due to the blood-brain barrier (BBB) (21), we wondered where and how immune cells invade the CNS to cause neuroinflammation. Through a series of experiments using EAE, we found that regional neural activation by the soleus muscle, which senses gravitational force, determines the location of immune cell entry into the CNS by altering the properties of blood vessels (3). To the best of our knowledge, this was the report that linked gravity and local inflammation through neuro-immune interactions.

The BBB is formed by tight cell-cell interactions between pericytes, endothelial cells, and astrocyte end-feet. Tight junctions are critical for separating the blood and cerebrospinal fluid (22). However, the barrier is not perfect, and it is widely recognized that immune cells can invade the CNS to cause autoimmune diseases such as MS. Recent studies have demonstrated the presence of CNS lymphatic vessels that connect to the cervical lymph nodes and may serve as an exit for immune cells from the CNS (23, 24). It is also known that breaching of the BBB is observed in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (25). Inflammation is a key component to modulate the integrity of the BBB, and pro-inflammatory cytokines including IL-1β, IL-17A, IFNγ, and TNFα are known to increase BBB permeability (26, 27).

Multiple sclerosis is a chronic inflammatory disease in the CNS that is characterized by impairments in sensory, motor, autonomic, and cognitive functions due to demyelination (28). Genetic factors strongly contribute to the pathogenesis of MS. Genome-wide association studies showed that certain alleles of major histocompatibility complex (MHC) class 2 and genes involved in $CD4^+$ T cell activation and survival are genetically associated with MS development (29–33). These genetic data strongly suggest that autoreactive $CD4^+$ T cells are essential for the pathogenesis of MS. Animal models of MS including EAE also demonstrated the pivotal role of autoreactive $CD4^+$ T cells (3, 4, 34–36). Thus, suppression of the differentiation and activation of $CD4^+$ T cells to autoreactive pathogenic T cells or blockade of their entry into the CNS could be a promising therapeutic strategy for MS.

Clinical symptoms of MS are dependent on the location of the demyelination, and MS patients show various damaged CNS sites. Given the wide distribution of a target autoantigen myelin in the white matter of the CNS (28, 33, 37), the heterogeneity of the damaged sites in each patient suggests an unknown mechanism. To identify the initial invasion site(s) of pathogenic $CD4^+$ T cells into the CNS, we used an adoptive transfer of CNS-autoreactive $CD4^+$ T cells to cause EAE (pathogenic $CD4^+$ T cells) and made whole-mount frozen sections of adult mice. These sections were made just before the onset of EAE clinical symptoms to find the first entry site of the pathogenic $CD4^+$

T cells. This analysis revealed that pathogenic CD4+ T cells mainly accumulated at the dorsal vessels of the L5 spinal cord but not in the upper spinal levels or brain at the preclinical time point. Compared to the dorsal vessels of the L1 spinal cord, many chemokines, including CCL20, which attracts IL-17-secreting type-17 CD4+ T (Th17) cells that have a vital pathogenic role in EAE (38, 39), are upregulated in the L5 dorsal vessels. Indeed, the L5 accumulation of pathogenic CD4+ T cells was inhibited by anti-CCL20 antibody treatment or by using CCL20 receptor-deficient CD4+ T cells. Even without EAE induction, chemokine levels were higher in the L5 dorsal vessels than in the L1 cord, although the levels were lower than those seen in the pathological condition of EAE. These results suggest that L5 dorsal vessels have some unique property under both normal and disease conditions. It is known that the L5 spinal level has the largest dorsal root ganglion (DRG) among spinal levels in both human and mice, and it is reported that L5 DRG neurons are connected to the soleus muscles, which are the main anti-gravity muscles and are activated even in steady state (40, 41). These facts led us to hypothesize that gravity stimulation to the soleus muscles may activate the L5 dorsal vessels to produce the chemokines that form the initial gateway for pathogenic CD4+ T cells. We examined this possibility using a ground test employed by the National Aeronautics and Space Administration (42). When normal mice were suspended by tail in a handstand position to free the hind legs from gravity stimulus, the chemokine expressions of the L5 dorsal vessels decreased and pathogenic CD4+ T cells failed to accumulate at L5. The cells instead invaded the cervical cords as if another gateway had been formed in response to the greater gravity stimulation on the arm muscles due to the tail suspension. Consistently, the tail suspension significantly decreased the expression levels of c-Fos, a marker for neural activation, in the L5 DRG. In addition, when the soleus muscles of the suspended mice were stimulated by weak electric pulses, chemokine and c-Fos expression levels and pathogenic CD4+ T cell accumulation at the L5 dorsal vessels were restored (3). These data suggest that regional sensory neural activation by gravity mediates local inflammation at L5 dorsal blood vessels, representing a novel neuro-immune interaction—the gravity-gateway reflex (14, 16–18, 43–46).

Since the autonomic nervous system mainly controls the function of blood vessels, its involvement in the gravity-gateway reflex was suggested. Neural activation based on c-Fos levels was higher in sympathetic ganglions of the L5 level than in those of L1. In addition, after tail suspension, blood flow speed at the L5 dorsal vessels became slower whereas in other blood vessels including L1 dorsal vessels, portal vein, or femoral artery it was not significantly affected. Furthermore, the slowed speed at the L5 dorsal vessels was recovered by electronic stimulation to the soleus muscles. These results suggest that autonomic nerves, particularly sympathetic nerves, could be involved. Functionally, pharmacological blockade of β-adrenergic receptors or chemical sympathectomy inhibited chemokine expressions and pathogenic CD4+ T cell accumulation at the L5 dorsal vessels and suppressed the severity of EAE (3). Thus, the gravity-gateway reflex involves local sensory-sympathetic communications for the gateway formation at the L5 dorsal vessels (**Figure 2**). These

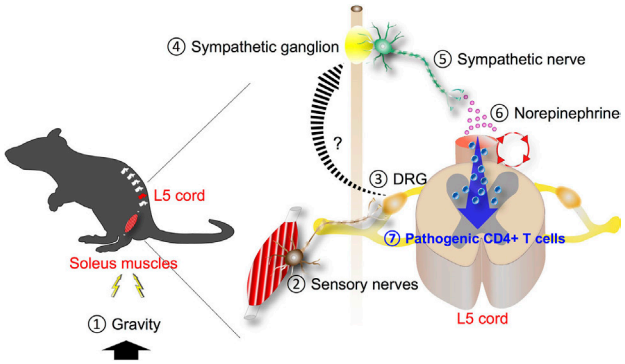

FIGURE 2 | Gravity-gateway reflex. Gravity stimulation (1) induces the activation of sensory nerves in the soleus muscles (2). The cell bodies of these neurons are present at the dorsal root ganglion (DRG) of the fifth lumbar (L5) spinal cord (3). Neural signals through the L5 DRG neurons are transmitted to sympathetic ganglion nearby (4) and activate sympathetic nerves (5), leading to NE secretion (6) at the L5 dorsal vessels. NE boosts the inflammation amplifier (red circle) there, causing chemokine production including CCL20 and recruitment of pathogenic CD4+ T cells that express CCL20 receptor, the receptor for CCL20 (7).

results represented the first example of a local neuro–immune interactions that regulate the condition of specific blood vessels to promote chemokine expression. Moreover, because gravity is an inevitable stimulus to land animals, the gravity-gateway reflex may have a physiological role that we have acquired during evolution. It also bears consideration for the health of astronauts and future space exploration.

Electric-Gateway Reflex

We next examined whether the gateway reflex is a specific property of the soleus–L5 axis and whether it can be artificially manipulated. We found that weak electric stimulation of neurons in different muscles of mice can create gateways at different levels of the spinal cord. For example, electric stimulation of the quadriceps, which are controlled by L3 DRG neurons, induced chemokine expressions at the L3 dorsal vessels. Moreover, stimulation of the forefoot muscles upregulated chemokines levels at the dorsal vessels in the cervical to thoracic spinal cords (**Figure 3**) (3). We termed this artificial control of the immune cell gateway in the BBB as the electric-gateway reflex. These results indicate that the gateway reflex can be artificially controlled and suggest a possible therapeutic target for CNS inflammatory diseases such as MS.

Pain-Gateway Reflex

We also investigated other sensory inputs on the gateway reflex. We focused on pain sensation because it is a tonic sensory stimulation (47, 48) and a common undesirable symptom that significantly compromises the quality of life in various diseases (49). It is reported that disease severity and pain occurrence in MS are positively correlated (50–52), and a change in pain sensation is described during EAE (53). In the adoptive transfer EAE model, we used recipient mice develop paralysis around 10 days after the transfer of pathogenic CD4+ T cells and then recover from

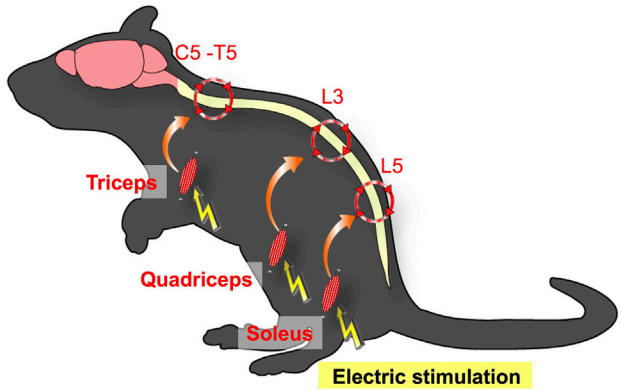

FIGURE 3 | Electric-gateway reflex. Neural activation using weak electric stimulation can induce the gateway reflex. Electric stimulation to the triceps induces chemokine upregulation at the dorsal vessels of the fifth cervical (C5) to fifth thoracic (T5) spinal cord *via* the inflammation amplifier activation (red circles). Likewise, electric stimulation of the quadriceps triggers chemokine upregulation at the L3 dorsal vessels, whereas the L5 gateway is formed by electric stimulation to the soleus muscles.

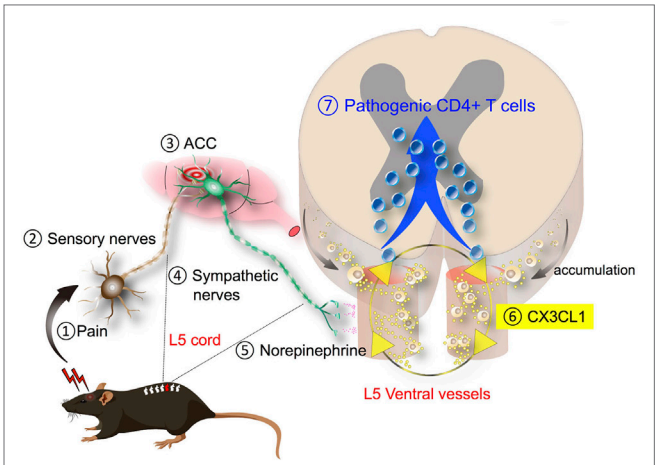

FIGURE 4 | Pain-gateway reflex. Pain-induced sensory stimulation (1, 2) induces the activation of the anterior cingulate cortex (ACC), a pain-processing region in the brain (3), which leads to the activation of specific sympathetic nerves (4), followed by norepinephrine (NE) secretion around the ventral blood vessels of the spinal cords (5). Because major histocompatibility complex (MHC) class $2^{Hi}CD11b^{+}$ monocytes are most abundant in the fifth lumbar spinal cord (L5), the L5 region is mainly affected after pain induction. NE secreted around the ventral blood vessels stimulates the production of chemokine CX3CL1 from MHC class $2^{Hi}CD11b^{+}$ monocytes. Because MHC class $2^{Hi}CD11b^{+}$ monocytes express CX3CL1 receptor CX3CR1, a positive loop is formed to recruit more of them (6). MHC class $2^{Hi}CD11b^{+}$ monocytes have an ability to antigen presentation to pathogenic $CD4^{+}$ T cells, leading to their invasion (7) and subsequently cause disease relapse.

EAE symptoms thereafter. To investigate how pain sensation affects EAE symptoms, we induced pain sensation in mice by surgical ligation of the middle branch of the trigeminal nerves, which are exclusively composed of sensory nerves (54). The pain induction at the time of pathogenic $CD4^{+}$ T cell transfer significantly deteriorated EAE symptoms. By contrast, treatment with pain medicines inhibited EAE development (4). Thus, pain is not only an index of the disease status but also plays a pathogenic role during EAE development. Since many MS patients show relapse, and MS patients showing higher disease scores more frequently claim pain (50, 51), we examined the impact of pain induction during a remission phase of EAE. As expected, mice recovered from EAE clearly relapsed into paralysis upon trigeminal nerve ligation as well as by injection of pain-causing chemicals such as substance P and capsaicin (4). As described earlier, the initial gateway is the dorsal vessels of the L5 spinal cord during the first episode of EAE (3). To identify the immune cell gateway during the pain-induced relapse, we performed immunohistological examination of EAE mice in remission phase (EAE-recovered mice). Although the appearance and motility of EAE-recovered mice were indistinguishable from normal healthy mice, high numbers of periphery-derived activated monocytes expressing high levels of MHC class 2 (MHC class $2^{Hi}CD11b^{+}$ cells) were found around the meningeal region of the L5 cord. Interestingly, pain induction directed these cells to the ventral but not dorsal vessels of the L5 cord. Furthermore, activation of NE signal transduction was evident around the L5 ventral vessels, and MHC class $2^{Hi}CD11b^{+}$ monocytes secreted CX3CL1 following NE stimulation at least *in vitro*, suggesting an autocrine/paracrine loop for MHC class $2^{Hi}CD11b^{+}$ monocyte accumulation. Following the monocyte accumulation at the L5 ventral vessels, pathogenic $CD4^{+}$ T cells in the blood flow invaded from the vessels to the spinal cord parenchyma. Thus, L5 ventral vessels are the gateway during pain-induced relapse in the EAE model (**Figure 4**). The pain-induced EAE relapse can be inhibited by genetic or pharmacological suppression of the pain sensory pathway or by chemical ablation of the sympathetic nerves, suggesting the involvement of a sensory–sympathetic communication akin to the gravity-gateway reflex (4). We hypothesize that systemic hormonal stress responses do not contribute to the pain-gateway reflex because transient stress loading by immobilization or by forced swimming did not induce EAE relapse despite equivalent levels of serum corticosterone, NE, and epinephrine to those induced by the pain model (4). These results suggest that various sensory stimuli trigger gateway reflexes at different regions of the CNS and implicate that pain control may be beneficial for preventing the relapse of neuroinflammation.

Stress-Gateway Reflex

It is well known that chronic stresses exacerbate illness. Chronic stress conditions often cause gastrointestinal (GI) diseases *via* the brain–gut axis. However, the molecular mechanism remains poorly understood. Because stresses are associated with neural activation involving brain regions such as the paraventricular nucleus (PVN), the dorsomedial nucleus of hypothalamus (DMH), the dorsal motor nucleus of the vagus nerve (DMX), and the vagal nerve pathway (55), we hypothesized that chronic stresses might activate another specific gateway reflex in the brain. Under chronic stress conditions such as sleep disturbance, we serendipitously found that EAE caused severe GI dysfunction with high mortality (56). While donor pathogenic $CD4^{+}$ T cells

accumulated at the L5 dorsal vessels under normal condition due to the gravity-gateway reflex (**Figure 2**), the stress condition directed them to invade at specific vessels of the boundary area to establish brain microinflammation. Therefore, chronic stresses can change the immune cell gateway from L5 to brain. Indeed, chemokine expressions in the specific vessels of the boundary area of the third ventricle (3V), dentate gyrus, and thalamus increased, whereas those in the L5 dorsal vessels decreased in EAE mice under chronic stress (56).

The resulting microinflammation in the brain specifically enhanced a novel neural pathway that includes the PVN, DMH, and vagal neurons. Neural tracing revealed neural connections, particularly TH^+ noradrenergic connections, from the PVN to the specific vessels and from the specific vessels to the DMH, which does not consist of TH^+ connections. Since the PVN is a principal integrator of stress signals, activation of the PVN is expected to influence specific blood vessels *via* the new identified neural circuit. Chemokine expressions including CCL5 at the specific vessels were upregulated in mice with stress only. In the presence of pathogenic $CD4^+$ T cells in stressed mice, these cells detect the chemokine upregulation, causing brain microinflammation at the specific vessels of the boundary region of the 3V, dentate gyrus, and thalamus, followed by the accumulation of various immune cells including periphery-derived MHC class $2^{Hi}CD11b^+$ cells (56). It is well known that an inflammatory response involves the production of various substances such as ATP that can serve as both inflammatory mediator and neurotransmitter (57, 58). We therefore tested an ATP receptor antagonist injected at the specific blood vessels of the boundary area of the 3V, dentate gyrus, and thalamus and found that neural activation in the DMH region was clearly inhibited, with a significant improvement in the mortality rate of EAE mice with stress. Furthermore, inhibition of the brain microinflammation by cytokine neutralization or blockage of the neural pathway also suppressed the GI dysfunction and improved mortality (56). We examined whether microinflammation at the specific blood vessels is sufficient for stress–EAE phenotypes. Importantly, direct injection of cytokines or ATP at the specific vessels established severe GI failure in mice with stress. These results suggest that microinflammation at the specific vessels turns a resting neural pathway on *via* ATP production, and the ATP-induced neural activation to the DMH region strongly enhances the stress response to cause severe GI damages *via* the DMX (**Figure 5**). These results reveal a direct association between brain microinflammation and GI homeostasis through a newly established specific neural pathway under chronic stress conditions (56). We defined this phenomenon as the stress-gateway reflex. Several studies suggest a cooccurrence of MS and inflammatory bowel diseases (59–63). Moreover, microinflammations in the brain can be observed in patients with neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, non-Alzheimer type dementia (64, 65), epilepsy (66), and psychological disorders (67). In addition, cerebral microbleeding is known to be a risk factor for dementia (68). We therefore suggest that brain microinflammations could act as a switch to turn on new neural pathways to control organ homeostasis including the brain itself, that several comorbidities might be influenced by the presence of brain microinflammation observed in many diseases, and that circulating $CD4^+$ T cells can be a biomarker and therapeutic target for stress-induced organ dysfunction.

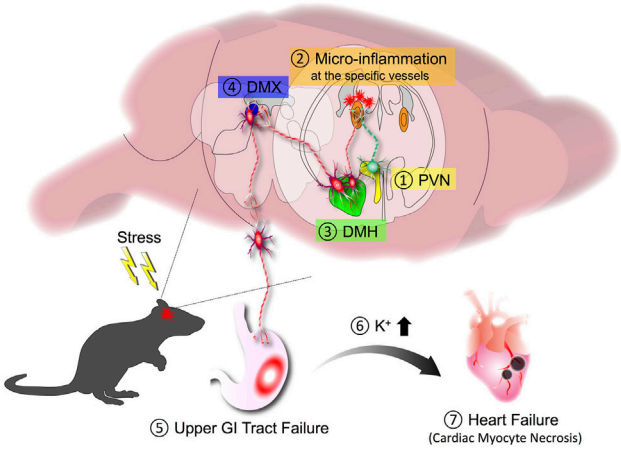

FIGURE 5 | Stress-gateway reflex. Under a chronic stress condition, experimental autoimmune encephalomyelitis mice showed high mortality that was associated with severe gastrointestinal (GI) failure. Chronic stress induces activation of the paraventricular nucleus (PVN) (1), which activates neurons connecting to the specific blood vessels of the boundary region of the third ventricle, dentate gyrus, and thalamus to establish microinflammation in the brain (2). The resulting microinflammation activates a resting neural pathway to the dorsomedial nucleus of hypothalamus (DMH) (3) and dorsal motor nucleus of the vagus nerve (DMX) (4) to cause severe upper GI tract failure (5). The upper GI tract failure results in the increase of potassium ions in the blood (6), which finally causes heart failure with cardiac myocyte necrosis (7).

In the gateway reflexes, neural activations induce chemokine production from vascular endothelial cells using the inflammation amplifier, a mechanism in which the concomitant activation of NF-κB and STATs in non-immune cells, such as fibroblasts and endothelial cells, leads to the hyperactivation of NF-κB (**Figure 6**). The inflammation amplifier is involved in the pathogenesis of several disease models in mice, and evidence of its activation has been demonstrated in human clinical specimens (69–74). Since neurotransmitters such as NE can promote NF-κB activity (75), they enhance the amplifier to further induce NF-κB target genes including chemokines (**Figure 6**) (3). Further details about the inflammation amplifier can be found in other review articles (69, 76).

A MYELOID SUBSET THAT MEDIATES THE GATEWAY REFLEX

The gateway reflex suggests artificial stimulation of the appropriate neurons can have a potential clinical application. Analogously, vagal nerve stimulation has been tested to modulate the inflammatory reflex in rheumatoid arthritis patients with positive results (20). Another possibility would be to target specific cell type(s) involved in the gateway reflex. During the study of the pain-gateway reflex, we found that periphery-derived MHC class

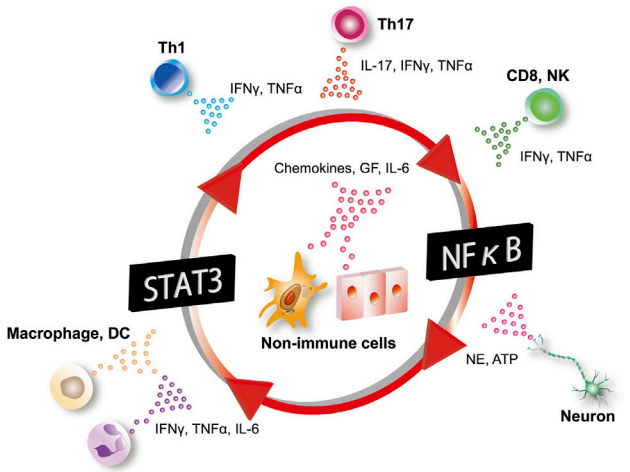

FIGURE 6 | Inflammation amplifier. In the inflammation amplifier, a concurrent activation of the transcription factors NF-κB and STATs in non-immune cells including vascular endothelial cells and fibroblasts induces a multiplier effect on the production of growth factors (GF), chemokines, and cytokines such as IL-6. Various factors activating NF-κB and STATs can operate the amplifier. IL-6 can act on non-immune cells to form an amplifying loop. Excessive production of chemokines and GF through activation of the inflammation amplifier play a key role in the pathogenesis of many chronic inflammatory diseases. DC, dendritic cells; NK, natural killer cells; NE, norepinephrine; Th, helper T cells.

2^{Hi}CD11b$^+$ monocytic myeloid lineage cells play an essential role in pain-induced EAE relapse (**Figure 4**), suggesting that these are a possible cellular target to control the gateway reflex. Local depletion of MHC class 2^{Hi}CD11b$^+$ monocytes in the CNS significantly suppressed EAE relapse by pain sensation. Systemic depletion of pathogenic CD4$^+$ T cells in EAE-recovered mice also inhibited relapse of EAE, but MHC class 2^{Hi}CD11b$^+$ monocytes still accumulated around the ventral vessels of the L5 cord. These data indicate that the migration of MHC class 2^{Hi}CD11b$^+$ monocytes to the L5 ventral vessels by the pain-gateway reflex precedes pathogenic CD4$^+$ T cell invasion and is a critical step for the relapse of EAE (4). Experiments using parabiosis showed that MHC class 2^{Hi}CD11b$^+$ monocytes are derived from the periphery, infiltrate the CNS during the first symptom of EAE, and have long life span in the CNS. Interestingly, the pharmacological blockade of *N*-methyl-D-aspartic acid (NMDA) receptor at the anterior cingulate cortex, where the pain-mediated sensory signal transits to a sympathetic signal, inhibited the pain-induced accumulation of MHC class 2^{Hi}CD11b$^+$ monocytes in EAE-recovered mice, while activating this neural pathway by the injection of a NMDA receptor agonist induced the accumulation of the cells even without pain induction (4). Similar periphery-derived MHC class 2^{Hi}CD11b$^+$ monocytic cells were also detected in the specific vessels of EAE mice under stress conditions (56). These results suggest that periphery-derived MHC class 2^{Hi}CD11b$^+$ monocytic cells are a unique myeloid subset that serves as an interface for neuro-immune interactions during the gateway reflex and is a potential cellular target for the treatment of inflammatory diseases in the CNS.

PERSPECTIVE

Growing evidence has demonstrated significant functions of specific regional neuro-immune interactions during inflammation and disease. As described in this review, vagus and splenic nerve-mediated control of specific subsets of CD4$^+$ T cells and macrophages that produce and respond to acetylcholine is a main axis of the inflammatory reflex (14–18, 77). For the gateway reflexes, different players including CNS-reactive pathogenic CD4$^+$ T cells and periphery-derived MHC class 2^{Hi}CD11b$^+$ monocytic cells drive the response (3, 4, 44, 45, 56). Recently, a pilot study was performed that stimulated the vagus nerve of seven Crohn's disease patients, and five patients showed deep remission at 6 months of follow-up without major side effects (78). In addition, Koopman et al. showed that vagus nerve stimulation in rheumatoid arthritis patients significantly improved disease severity with reduced TNF production (20). To activate specific neurons from the body surface to induce desired effects, elucidation of the precise neural networks for both reflexes is anticipated. Although identification of the detailed neural pathways is challenging, recently developed imaging techniques that use a tissue decoloring reagent, CUBIC (79–81), in conjunction with various neural tracers and transgenic mice will help. Further investigation of the gateway reflexes in response to various stimuli will also help identify novel functional neural connections that govern organ homeostasis or pathogenesis associated with local inflammation. Mapping of the neural connections and identifying more examples of gateway reflexes could lead to the elucidation of physiological functions of the gateway reflexes, which are currently not clear. In addition, whether the MHC class 2^{Hi}CD11b$^+$ monocytic lineage and brain microinflammation-inducing CD4$^+$ T cells are present in humans is an unanswered question. To do so, specific markers for these cell types are needed.

Because neuronal circuits run throughout the whole body and because immune cells (10, 82–84) and non-immune cells (3, 4, 56) can secrete and respond to neurotransmitters, specific local neuro-immune interactions such as the gateway reflex and inflammatory reflex have tremendous therapeutic potential for the treatment of various diseases without any major systemic side effects.

AUTHOR CONTRIBUTIONS

All authors wrote and elaborated manuscript and figures.

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REVIEW
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The Vagus Nerve in the Neuro-Immune Axis: Implications in the Pathology of the Gastrointestinal Tract

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The vagus nerve (VN) is the longest nerve of the organism and a major component of the parasympathetic nervous system which constitutes the autonomic nervous system (ANS), with the sympathetic nervous system. There is classically an equilibrium between the sympathetic and parasympathetic nervous systems which is responsible for the maintenance of homeostasis. An imbalance of the ANS is observed in various pathologic conditions. The VN, a mixed nerve with 4/5 afferent and 1/5 efferent fibers, is a key component of the neuro-immune and brain-gut axes through a bidirectional communication between the brain and the gastrointestinal (GI) tract. A dual anti-inflammatory role of the VN is observed using either vagal afferents, targeting the hypothalamic–pituitary–adrenal axis, or vagal efferents, targeting the cholinergic anti-inflammatory pathway. The sympathetic nervous system and the VN act in synergy, through the splenic nerve, to inhibit the release of tumor necrosis factor-alpha (TNFα) by macrophages of the peripheral tissues and the spleen. Because of its anti-inflammatory effect, the VN is a therapeutic target in the treatment of chronic inflammatory disorders where TNFα is a key component. In this review, we will focus on the anti-inflammatory role of the VN in inflammatory bowel diseases (IBD). The anti-inflammatory properties of the VN could be targeted pharmacologically, with enteral nutrition, by VN stimulation (VNS), with complementary medicines or by physical exercise. VNS is one of the alternative treatments for drug resistant epilepsy and depression and one might think that VNS could be used as a non-drug therapy to treat inflammatory disorders of the GI tract, such as IBD, irritable bowel syndrome, and postoperative ileus, which are all characterized by a blunted autonomic balance with a decreased vagal tone.

Keywords: vagus nerve, vagus nerve stimulation, cholinergic anti-inflammatory pathway, neuro-immune axis, splenic nerve

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INTRODUCTION

The vagus nerve (VN), the longest nerve of the organism, makes the link between the central nervous system and the body by innervating major visceral organs such as the heart, the lungs, and the gastrointestinal (GI) tract. The VN is a mixed nerve with 20% efferent and 80% afferent fibers (1), and a major component of the parasympathetic nervous system which composes, with the sympathetic nervous system, the autonomic nervous system (ANS). The sympathetic and parasympathetic nervous

systems are classically balanced for maintaining homeostasis. This balance of the ANS is disrupted in various pathologies such as irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD), rheumatoid arthritis (RA), and others, and such an imbalance could also be a predictor of various neuro-immune disorders (2, 3). In particular, an autonomic dysfunction, as represented by a low parasympathetic activity, precedes the development of chronic inflammatory disorders such as RA (4). Consequently, an autonomic dysfunction could be involved in the etiopathogenesis of inflammatory disorders rather than being the consequence of chronic inflammation. The modulation of the ANS, in particular by targeting the VN, is able to improve various pathological conditions such as inflammatory disorders, including IBD, RA, obesity, and pain (5). Such a modulation of the VN is possible through pharmacological manipulation, VN stimulation (VNS), nutritional therapies, physical exercise, and complementary medicines. The VN classically does not innervate lymphoid organs; this role is dedicated to the sympathetic nervous system (6). However, the VN is involved in the neuro-immune axis both through its afferent and efferent fibers. Indeed, the VN stimulates the hypothalamic–pituitary–adrenal (HPA) axis through its afferent fibers to release glucocorticoids by the adrenal glands (7). The VN is also involved in the cholinergic anti-inflammatory pathway (CAP) through a vago-vagal reflex involving a brainstem integrated communication between vagal afferent and efferent fibers i.e., the inflammatory reflex (8, 9). The sympathetic nervous system and the VN interact both through a vago-sympathetic pathway involving vagal afferent fibers (10) and a vago-splenic pathway through vagal efferent fibers (11). Consequently, the VN is at the crossroad of neuro-immune interactions and by stimulating the VN, it is possible to treat various inflammatory disorders of the organism.

In the present manuscript, we will *first*, describe the anatomy of the VN, *second*, characterize the interactions of the VN with the HPA axis and the CAP and the sympathetic nervous system, *third*, explore the interest of therapeutic manipulation of the VN for anti-inflammatory properties through pharmacological activation, VNS, complementary medicines (acupuncture, hypnosis, mindfulness), enteral nutrition, physical exercise, and *fourth*, focus on the role of VNS in the modulation of inflammatory disorder conditions and particularly of the GI tract, such as IBS, IBD, and postoperative ileus (POI).

ANATOMY OF THE VN

The VN innervates all the GI tract of the rat, except for the rectum (12). In contrast, in human, the GI tract innervation by the VN is debated. For some authors, the VN innervates the digestive tract until the splenic flexure of the colon (13) and the sacral parasympathetic nucleus innervates the rest of the gut through the pelvic nerves; the densest innervation is provided to the stomach. However, the VN could innervate all the digestive tract in human (14). The VN is composed of 80% afferent fibers conveying taste, visceral and somatic information and 20% efferent fibers involved in the control of motility and secretion of the GI as well as cardiac parasympathetic tone (15) and the CAP (8).

Preganglionic neurons of vagal efferents originate in the dorsal motor nucleus of the vagus (DMNV), below the nucleus tractus solitarius (NTS) where vagal afferents project to. A viscerotopic distribution has been described in the rat DMNV such that lateral neurons innervate the stomach while medial neurons innervate the colon (16). Preganglionic neurons are connected with post-ganglionic neurons of the enteric nervous system in the GI tract. Acetylcholine (ACh) is the neuromediator released at both ends of these pre- and post-ganglionic neurons which binds to nicotinic receptors and nicotinic or muscarinic receptors, respectively. The VN is not in direct contact with the intestinal lamina propria (16) but through these enteric neurons (17) which are the effectors of the VN to regulate gut immunity (18).

Vagal afferent fibers originate from the different intestinal layers with their cell bodies located in the nodose ganglia. They end in the NTS according to a rostro-caudal viscerotopic representation (19), and then to the area postrema. The DMNV forms, with the NTS and area postrema, the dorsal vagal complex of the brainstem, a major reflex center of the ANS. Indeed, the activation of vagal afferents generates several coordinated responses (autonomic, endocrine, and behavioral) *via* central pathways involving the dorsal vagal complex. Viscero-sensory informations coming from the NTS to the DMNV influence vagal efferents at the origin of vago-vagal reflexes (20). In addition, the NTS is a relay for these peripheral informations to reach numerous brain areas (21) which compose the central autonomic network (CAN) (22) such as the locus coeruleus (LC), the parabrachial (PB) nucleus the periventricular nucleus of the thalamus, the central nucleus of the amygdala, the paraventricular nucleus of the hypothalamus (PVH), the medial preoptic area, the arcuate nucleus of the hypothalamus, and the ventrolateral medulla (A1-C1 catecholaminergic nuclei) at the origin of an autonomic, behavioral, and endocrine response. The NTS also directly modulates the LC and its projections (23). The rostroventrolateral medulla is one of the two major sources of projections to the LC (24). The latter project to numerous areas of the cortex involved in stress reactions but also in emotional disorders (25). The PVH projects to the bed nucleus of the stria terminalis, the dorsomedial and arcuate hypothalamic nuclei, the medial preoptic area, the periventricular nucleus of the thalamus, the PB region, and the nucleus tegmenti dorsalis lateralis (26). The PB nucleus in return projects to the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the PVH (27). The PVH projects directly to the NTS (26), thus creating a feedback loop with the forebrain. Consequently, visceral information (e.g., nutrient sensing) driven by the VN is integrated in the CAN involved in the functioning of the ANS and the HPA axis response. The VN is involved in the interoceptive awareness where the insula cortex plays a central role (28). A perturbation of this interoception is observed in diseases of the digestive tract such as IBS but also IBD. Indeed, alexithymia (29) is observed in both of them (30–32).

THE VN AND THE NEURO-IMMUNE AXIS

The VN is a key component of the neuro-immune axis both through its afferent and efferent fibers. The role of vagal afferents

was first described by Harris (7) in the regulation of the HPA axis. Indeed, peripheral administration of lipopolysaccharides (LPS), classically used as an experimental model of septic shock, induces the release of interleukin (IL)-1β, a pro-inflammatory cytokine, and finally activates vagal afferents through IL-1 receptors (33). This effect is prevented by vagotomy (34) and works in a dose and receptor-dependent fashion (35). Vagal afferents activate NTS neurons from the A2 noradrenergic group which project to corticotrophin-releasing factor (CRF) neurons of the parvo-cellular PVH. CRF then induces the release of adrenocorticotropic hormone by the pituitary to stimulate the release of glucocorticoids by the adrenal glands to inhibit peripheral inflammation, i.e., the HPA axis.

In addition to this vagal afferent anti-inflammatory pathway, a second one, described in 2000 by the group of Tracey, involves vagal efferents (36). This group showed that stimulation of the distal end of the VN, i.e., vagal efferents, prevented a LPS-septic shock in rats. VNS had an anti-TNFα effect since liver and blood tumor necrosis factor-alpha (TNFα) levels were dampened. The release of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-18 in LPS-stimulated human macrophages was decreased by the release of ACh by the VN. These authors called this pathway "The CAP" (8) (**Figure 1**) assimilated to an "inflammatory reflex," i.e., a vago-vagal reflex where the activation of vagal afferents by LPS-stimulated vagal efferents after central integration in the dorsal vagal complex. This group has also identified the α7 nicotinic ACh receptors (α7nAChR) of macrophages involved in this effect (37). de Jonge et al. (38) characterized the cellular mechanistic of this pathway involving α7 subunit-mediated Jak2-STAT3 activation of macrophages and Sun et al. (39) showed that microRNA-124 is responsible of the CAP action by the inhibition of pro-inflammatory cytokines production. The VN is not directly connected with gut resident macrophages but interacts with enteric neurons expressing nNOS, VIP, and ChAT and located within the muscularis next to these macrophages expressing the α7nAChR (40, 41).

The VN has thus a dual anti-inflammatory action both *via* its afferent and efferent fibers activating the HPA axis and the CAP, respectively. Another anti-inflammatory pathway is the vago-splenic pathway.

THE VAGO-SPLENIC PATHWAY

The group of Tracey also described a vago-splenic pathway, i.e., a vago-sympathetic pathway through the spleen (11). Classically the parasympathetic (i.e., the VN in the present case) and the sympathetic nervous systems have an opposite effect. However, in the vago-splenic pathway, this effect is synergistic through a connection between the VN and the splenic nerve, a sympathetic nerve issued from the celiac ganglion (42), to activate the splenic nerve through the effect of ACh on α7nAChR. The final effect is the inhibition of TNFα release by the spleen (43). A non-neuronal cholinergic pathway is involved in this effect by contrast to the vagal neuronal cholinergic pathway. Indeed, norepinephrine, released by the splenic nerve, binds to β2 receptors of T-lymphocytes of the spleen which release ACh that links to α7nAChR of macrophages to inhibit the release of TNFα by these macrophages (44). These T-lymphocytes are located in the white pulp of the spleen, particularly the central region receiving a dense noradrenergic innervation (45). By comparison to the CAP, there is an intermediate step with a neuro-immune connection involving the splenic nerve and T-lymphocytes. However, the existence of this pathway is still controversial (46) since some authors argue in favor of a direct sympathetic mechanism (47) (see **Figure 1**). In contrast, the group of Ghia showed that intracerebroventricular injection of a M1 muscarinic ACh receptor agonist activated the CAP; this effect was reversed by vagotomy or splenic neurectomy (48). The same group showed that administration of galantamine, a central ACh-esterase inhibitor activated the CAP and this effect was suppressed by vagotomy, splenic neurectomy, or splenectomy (49). However, a lack of evidence for cholinergic innervation of the rat spleen was reported by Bellinger et al. (50). Martelli et al. (51) argue that the efferent mediator of the CAP is not the VN but the sympathetic nerve, i.e., the splenic nerve. Indeed, they showed that vagotomy has no effect on the LPS-induced TNFα response while both splenic and splanchnic nerves were LPS-activated and suppressed by splanchnicectomy, increasing TNFα levels (46). They evoked a splanchnic anti-inflammatory pathway. In both works of the group of Tracey and Martelli, the model used to activate the CAP and/or the splanchnic pathway was a septic shock induced by LPS which is rather different than other inflammatory conditions in experimental models of IBD and RA. However, both the role of a CAP and a splenic anti-inflammatory pathway are not incompatible when considering a vago-sympathetic pathway involving vagal afferents to the CAN and then descending pathways from the CAN to activate sympathetic nerves.

The sympathetic innervation of the spleen modulates the cellular and humoral immune responses of this lymphoid organ (52–56). Actually, the noradrenergic fibers innervating the spleen (42, 57) are in close contact with immune cells of the white pulp expressing adrenergic receptors (58, 59). The splenic preganglionic neurons located in the thoracic and rostral lumbar spinal cord (60) are controlled by a specific supra-spinal complex circuitry involved in the regulation of neural–immune interactions in the spleen. The VN is able to modulate the sympathetic nervous system after central integration of its afferents in the CAN which is then able to modulate the sympathetic nerves, such as the splenic nerve, through descending pathways from the CAN, i.e., a vago-sympathetic pathway.

THE VAGO-SYMPATHETIC PATHWAY

As described above, vagal afferents end in the NTS and from there activate the CAN which in return is able to modulate the ANS through descending pathways targeting the DMNV and the tractus intermediolateralis in the spinal cord at the origin of vagal and sympathetic efferents respectively. Five brain nuclei of the CAN (i.e., PVH, the A5 noradrenergic group, the caudal raphe region, the rostral ventrolateral medulla, and the ventromedial medulla) modulate the sympathetic outflow (61–63) by innervating preganglionic sympathetic neurons of the intermediolateral cell column in the spinal cord. Hence, the VN could induce a non-direct anti-inflammatory reflex by enhancing the

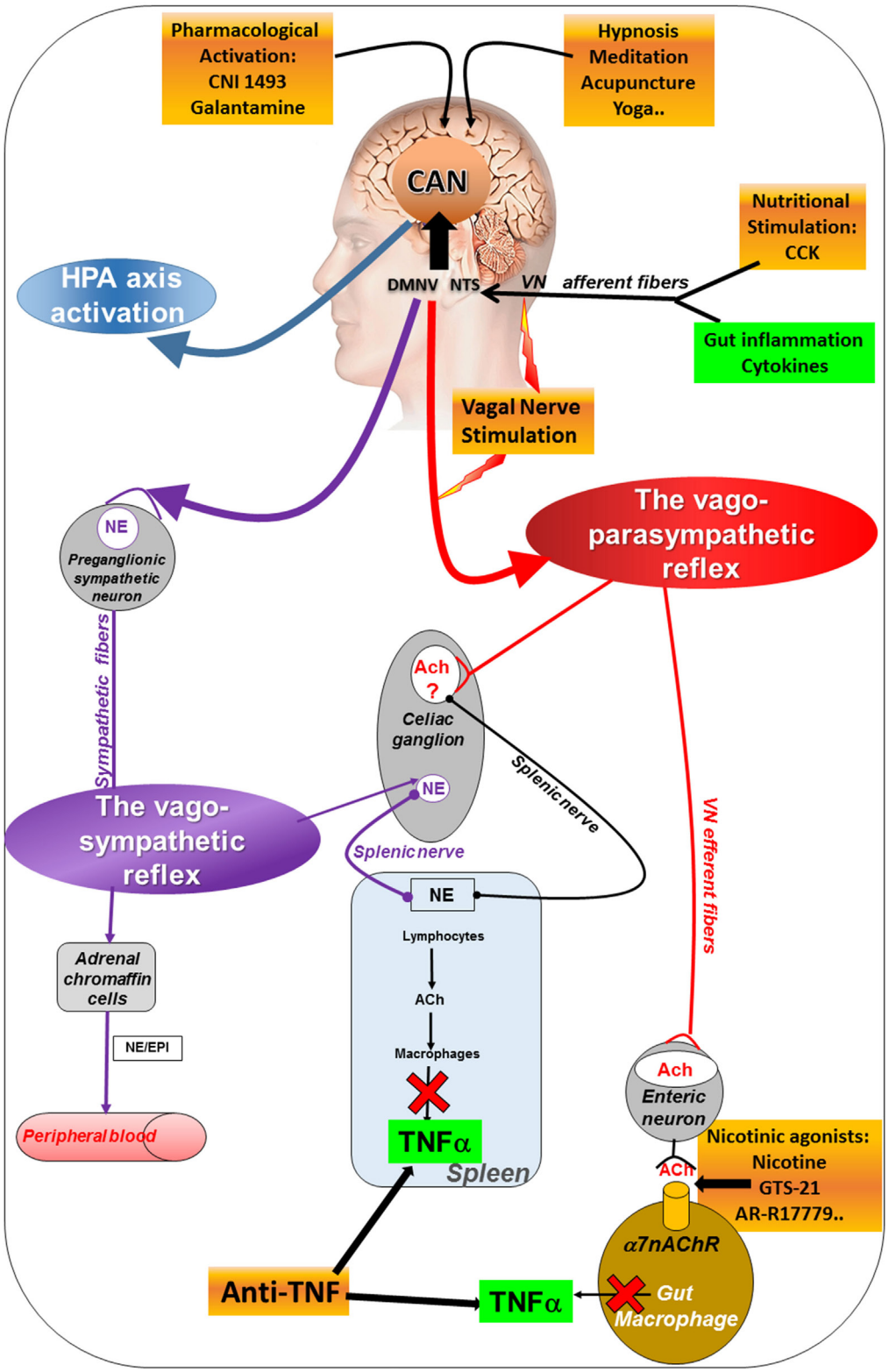

FIGURE 1 | Different pathways of the anti-inflammatory properties of the VN: activation of the HPA axis (blue) through vagal afferents, the cholinergic anti-inflammatory pathway through vago-parasympathetic (red) and sympathetic (purple) reflexes. Targeting the VN for its anti-inflammatory properties (orange) in chronic inflammatory diseases such as inflammatory bowel disease appears as potentially effective therapeutics. Ach, acetylcholine; CAN, central autonomic network; CCK, cholecystokinin; DMNV, dorsal motor nucleus of the vagus nerve; EPI, epinephrine; HPA, hypothalamic–pituitary–adrenal; NE, norepinephrine; NTS, nucleus tractus solitarius; TNFα, tumor necrosis factor-alpha; VN, vagus nerve; α7nAChR, alpha7nicotinic acetylcholine receptor.

sympathetic outflow. Among these brain structures, the role of the C1 adrenergic group has been recently highlighted by Abe et al. (64) who showed that these adrenergic neurons are involved in the stress protective effect in renal ischemia-reperfusion injury through a sympathetic rather than a vagal pathway. This group had previously shown that activation of vagal afferents or efferents in mice 24 h before injury markedly reduced acute kidney inflammation and TNFα plasma level. This effect was suppressed by splenectomy and was mediated by α7nAChR-positive splenocytes (65). The PVH, through its efferent projections to the DMNV and the spinal sympathetic preganglionic neurons is also able to modulate the ANS. For example, stress through CRF of the PVH, is able to inhibit the DMNV, i.e., vagal efferents, and activate the sympathetic nervous system, i.e., sympathetic efferents (66). Deng et al. (67) have recently shown that chemical stimulation of the hypothalamus protects against colitis in rats through a key role of PVN, NTS and VN. The A5 noradrenergic nucleus of the ventrolateral pons targets almost exclusively the spinal intermediolateral column (68) and is involved in the regulation of visceral sympathetic tone in rodents (69). A5 receives inputs from the C1 neurons (70). The effect of a stimulus on the activity of sympathetic nerves depends on their type fibers composition (71). The relative importance of each of these five regions in the control of the sympathetic outflow may differ. For example, for the spleen, A5 > rostroventrolateral medulla > PVH (71). After pseudorabies injection into the spleen, the A5 region is among the first areas to become infected. Consequently, this region is involved in the response of all sympathetic-innervated organs. A5 neurons must be connected to multiple sympathetic targets. Additional areas may selectively innervate sympathetic preganglionic neurons such as (i) the Barrington's nucleus exclusively involved in the control of the parasympathetic outflow, (ii) the LC involved in stress and contributing to the generalized sympatho-adrenal activation in response to stressful stimuli, (iii) the periaqueductal gray, lateral hypothalamus, A7 region, NTS, Edinger-Westphal nucleus, pedunculopontine tegmental nucleus, C3 group, caudal ventrolateral medulla, and area postrema (72). Neurons in the rostral ventrolateral medulla increase their activity in association with increases in sympathetic vasomotor reactions (73). All these observations reveal that sympathetic outflow is differentially regulated by supra-spinal areas, without a clearly identified mechanism. Moreover, some areas coordinate global visceral responses (74) thus making it difficult to target specific circuits.

HOW TO TARGET THE VN FOR ANTI-INFLAMMATORY PROPERTIES

The anti-inflammatory properties of the VN could be targeted pharmacologically, with enteral nutrition, by VNS, with complementary medicines or by physical exercise.

Pharmacological Stimulation of the CAP

Pharmacological stimulation can be obtained by targeting the CAP either centrally or peripherally.

Galantamine, a cholinesterase blocker and a nicotinic receptor agonist, including α7nAChR, is able to cross the blood–brain barrier and activates the central cholinergic pathway thus stimulating VN efferents (75). This drug is used in the treatment of Alzheimer's disease. Galantamine dramatically decreases circulating TNFα and IL-6 and improves survival in a murine endotoxemia model (75). Thus, galantamine could be used as an immune suppressive drug. To our knowledge, galantamine has only been used in experimental inflammation but not in clinical research. In the same way, CNI-1493 inhibits the p38 MAPK pathway of the TNFα release (76, 77). Central injection of CNI-1493 during endotoxemia significantly reduced serum TNFα levels and this effect is mediated through the VN (9). In a clinical trial, Crohn's disease (CD) patients who were treated with two doses of CNI-1493 for 2 weeks presented a clinical remission and an endoscopic improvement up to 45% of the patients included (78).

Peripheral α7nAChR can be targeted by agonists such as GTS-21 that was used in a double-blind placebo control trial in experimental human endotoxemia. Healthy volunteers after either GTS-21 or placebo received a low dose of LPS. GTS-21-treated group exhibited lower plasma TNFα, IL-6, and IL-1ra levels compared to placebo (79). In an experimental pancreatitis in mice, pretreatment with GTS-21 significantly decreased pancreatitis severity (80). AR-R17779, another α7nAChR agonist, prevented a mouse model of POI (81).

Nutritional Stimulation of the CAP

In a model of hemorrhagic shock, enteral nutrition with a high-fat diet induces the release of cholecystokinin (CCK), known to activate CCK1 receptors of vagal afferents, and dampens the inflammatory response (TNFα, IL-6) through a vago-vagal anti-inflammatory reflex (82). In the same study, CCK, vagotomy and nicotinic receptor antagonists prevented the protective effect of high-fat enteral nutrition on intestinal permeability (82). Mucosal mast cells are targets of the nutritional anti-inflammatory vagal reflex since mucosal mast cell degranulation was prevented by lipid-rich enteral feeding (83). Consequently, high-fat enteral nutrition could be used in the treatment of IBD where TNFα and intestinal barrier dysfunction are prominent. Enteral feeding, classically used in the treatment of a flare of IBD, has shown its efficacy to induce clinical remission in CD (84).

Complementary Medicines

Inflammatory bowel diseases are chronic debilitating diseases with an impact on quality of life and treatments are not always efficient and not devoid of side effects. Consequently, patients often use complementary medicines. Recently, Cramer et al. (85) assessed the efficacy and safety of yoga performed 90 min per week for 12 weeks for improving quality of life in UC patients in clinical remission. By comparison to the written self-care advice group (controls, $n = 38$), the yoga group ($n = 39$) had significantly higher disease-specific quality of life at 12 and 24 weeks of follow-up and disease activity was lower at 24 weeks. Gut-directed hypnotherapy is well known to improve IBS patients (86). Keefer et al. (87) performed seven sessions of gut-directed hypnosis in 26 UC patients in clinical remission vs 29 patients with attention control; the patients were follow-up for 1 year. Patients in the hypnosis group stayed significantly longer in remission at one

year than the control group (68 vs 40%). No significant effect has been observed for other psychological factors (quality of life, medication adherence, perceived stress). One mechanism through which complementary medicines may improve IBD could be the activation of the CAP. Acupuncture and meditation reduce both heart rate and inflammatory cytokine release. This effect is mediated by the increase of vagal tone (88). Acupuncture is able to decrease TNFα release following LPS administration in mouse (89). Acupuncture is associated with a down regulation of TNFα synthesis in the spleen that was reversed by splenic neurectomy and vagotomy. Hypnosis modifies heart rate variability (HRV) by enhancing parasympathetic activity and reducing sympathetic tone (90). Yoga (91) and mindfulness meditation (92) increase vagal activity. Consequently, these complementary medicines may be of interest in the treatment of IBD patients *via* the CAP.

VN Stimulation

In 1880s, Corning JL (93) was the first to use VNS for the treatment of seizures. The technique was then forgotten but reintroduced in 1938 by Bailey and Bremer (94). In 1990, the first VNS for the treatment of pharmacoresistant epilepsy was introduced in human (95) and VNS was approved by the US Food and Drug Administration (FDA) for this indication in 1994 and in 1997 for Europe. In 2005, the FDA approved VNS for the treatment of pharmacoresistant depression (96, 97). Presently, ~100,000 patients have been treated by VNS for epilepsy and ~5,000 for depression (Livanova, Houston, TX, USA).

The antiepileptic and antidepressive effects of VNS can be easily explained by the widespread projections of the VN in the brain from its first relay in the NTS. The mechanism of action of VNS is still not well understood but data argue for a role of the LC, thalamus, hippocampus, periaqueductal gray, and the neocortex (98). If the role of vagal afferent C-fibers was evoked in the antiepileptic effect of VNS, their alteration by capsaicin did not suppress the effect, arguing for a role of vagal A- and B-fibers (99). Five parameters of VNS are classically used: intensity (0.5–3.5 mA), frequency (20–30 Hz), pulse width (250–500 μs), and duty cycle of 30 s ON and 5 min OFF. Frequencies of 2–300 Hz induced electroencephalographic desynchronization of the "encéphale isolé" cat that was dampened by a ligature of the cervical end of the VN (100) thus in favor for a role of vagal afferent fibers. VNS effectiveness is frequency-dependent (101) up to the maximum threshold of 50 Hz beyond which a damage of the VN is induced (102). In rats, VNS (stimulation parameters used for epilepsy) induces neuronal activation in brain area involved in seizures initiation (103). In human, brain imaging studies reported modifications in regions receiving VN afferent supra-medullar projections (104). VNS is a slow-acting therapy since a seizure reduction appears in 50% of patients after 2 years (105). Elliott et al. (106) showed in 65 epileptic patients with a 10-year mean duration of VNS a time-dependent reduction in seizures. Indeed, the positive effect of VNS at 6 months and 1, 2, 4, 6, 8, and 10 years was 35.7, 52.1, 58.3, 60.4, 65.7, 75.5, and 75.5%, respectively.

Vagus nerve stimulation can be applied invasively or non-invasively through the skin. Invasive VNS is classically performed under general anesthesia by a neurosurgeon and an electrode is wrapped around the left cervical VN in the neck connected subcutaneously by a cable to a pulse generator located in the left chest wall (107). The implantation lasts ~1 h. VNS is classically performed onto the left VN which innervates the atrioventricular node of the heart while the right VN innervates the sinoatrial node thus with a weaker influence on the heart rate (108). The VNS device is manufactured by Livanova, a merger of Cyberonics and Sorin (Houston, TX, USA), and composed of a pair of helical electrodes (2 or 3 mm diameter), a battery-powered generator, a tunneling tool, software and programming tools (www.livanova.com/). The price of the generator pulse (model 102) plus the electrode (model 302) is ~9,300 €. Safety and tolerability were demonstrated for implantable VNS (101). The minor adverse events which are classically reported by the patients are: voice alteration, cough, dyspnea, paresthesia, nausea, headache and pain; these adverse events decline over time and are easily controlled by reducing stimulation intensity (109). The battery life depends on the frequency of stimulation used and is longer for low frequency (5–10 Hz), e.g., ~5–10 years, than high frequency (20–30 Hz).

Based on the concept that the CAP involves parasympathetic outflow of the vagal nerve, VNS is performed at the lowest frequencies (1–5–10 Hz) to produce its anti-inflammatory effect. Borovikova et al. (36) performed low frequency (1 Hz) VNS in rats with cervical vagotomy and stimulated the distal end cut of the VN thus stimulating vagal efferents. Bernik et al. (110), who performed VNS of the left or right VN in anesthetized rats, demonstrated that a 20 min-stimulation prevented endotoxin-induced hypotension.

Non-invasive VNS (n-VNS) does not need surgical implantation and improves the safety and tolerability of VNS. Transcutaneous auricular VNS (ta-VNS) is one of these techniques. Indeed, the VN includes a sensory "auricular" branch that innervates exclusively the cymba concha of the external ear (111) and projects to the NTS in cats (112) and humans (113). ta-VNS produces the same cognitive and behavioral effects than VNS (114). When performed at 25 Hz in healthy adults, it affects the vagal central projections, compared to a control stimulation in the earlobe (113). The close anatomical connection between auricular concha, VN, NTS, and DMNV can thus explain the auricular-vagal reflex. Consequently, ta-VNS could activate the anti-inflammatory pathway. In agreement with this neuroanatomical concept, ta-VNS suppresses LPS-induced inflammatory responses *via* α7nAChR in rats (115) and this effect was suppressed after vagotomy or with α7nAChR antagonist injection.

Presently, there are two n-VNS devices that are used for epilepsy, depression, and headache but which could also be used in inflammatory disorders of the GI tract such as IBD, IBS, and POI as well as others. The Cerbomed device called NEMOS (Erlangen, Germany) uses an intra-auricular electrode (like an earpiece) to stimulate the vagal auricular branch (116) and has received the European clearance in 2011 for the indication epilepsy. This device is available in Austria, Germany, Italy, Switzerland, and UK. The optimal stimulation is chosen by the patients based on the intensity to feel a non-painful stinging with a recommended stimulation duration of 4 h per day. A 70% reduction seizure frequency was observed after 9 months of ta-VNS (116) and

a 43% reduction has been observed after 8 weeks in another study (117). ta-VNS was shown to increase HRV and reduce sympathetic outflow in controls (118). The second device is referred as GammaCore (electroCore LLC, Basking Ridge, NJ, USA) and comprises a portable stimulator and two stainless steel round disks functioning as skin contact surfaces that deliver a locked, low-voltage electrical signal to the cervical vagal nerve; each stimulation cycle lasts 120 s. An improvement of headache was reported in 48% of patients (119). In another study, mean pain scores were significantly reduced at 2 h from baseline in patients with chronic migraine (120). GammaCore is presently evaluated in controlled trials in North America and EU in patients with primary headache disorders. n-VNS with the Gammacore system decreases whole blood culture-derived cytokines and chemokines in healthy volunteers (121). No significant serious device-related adverse events have been reported with NEMOS and Gammacore. By comparison to invasive VNS, n-VNS has the disadvantage of its compliance which is an important problem in the treatment of chronic inflammatory diseases.

Physical Exercise

An imbalance of the ANS, with low vagal and high sympathetic activities, correlates with numerous pathological conditions such as arrhythmia, heart failure, and hypertension and ischemia/reperfusion injury. Cardiovascular morbidity and mortality and inflammation are all decreased by high levels of cardiorespiratory trainings (122, 123). There is a negative correlation between cardiorespiratory fitness and cardiovascular events, partly mediated by inflammatory factors (124). The ANS is known to affect the relation between cardiorespiratory fitness and inflammation in middle-aged men. Then, physical activity and exercise training may exert a stimulatory effect on the CAP since RR variability is inversely related to inflammatory markers (125). Regular physical exercise induces an increase in resting vagal tone (126) and increases central 5-HT synthesis and central 5-HT increases vagal modulation in conscious rats (127).

VN IN THE MODULATION OF INFLAMMATORY DISORDER CONDITIONS

Based on its activation of the HPA axis and the CAP, the VN has the ability to modulate inflammatory conditions. Experimental and more recently clinical data involving pilot studies are available for this effect in the domain of IBD, RA, and POI. In the next lines, we will focus on GI inflammatory disorders such as IBD, IBS, and POI.

Chronic Inflammatory Bowel Disorders

Inflammatory bowel diseases are classically represented by CD and ulcerative colitis (UC). CD involves all the digestive tract and ano-perineal region while UC involves the recto-colon. IBD begin between 15 and 30 years and are characterized by alternation of flares and remissions. During flares, patients have several intestinal and extra-intestinal symptoms such as abdominal pain, diarrhea, skin, eyes, or joints inflammation thus explaining their significant impact on the quality of life of IBD patients. Both CD and UC are heterogeneous in their natural history (128). About 1.5 million Americans and 2.2 million Europeans are affected by IBD (129) and there is an increase of the incidence and prevalence of IBD due to the "Westernization" of our lifestyle. Immunologic, genetic, and environmental factors are involved in the pathophysiology of IBD (130). Experimental and clinical data seem to show a role of stress in the pathophysiology of IBD (131). Classically, stress increases intestinal permeability, modify intestinal microbiota and immunity which are factors involved in the pathophysiology of IBD. The VN is involved in the stress effects on the digestive tract. Indeed, stress classically inhibits the VN and stimulates the sympathetic nervous system (66). Chronic stress instead of acute stress is more involved in the pathophysiology of IBD as well as others GI disorders such as IBS (132). Stress induces an imbalanced ANS as reported in IBD with a blunted sympathetic activity in CD (133) and a vagal dysfunction in UC (134). We previously reported a relationship, in IBD patients, between an imbalanced ANS, psychological adjustment (3) and pro-inflammatory profiles (135). Presently, standard treatment of IBD patients is represented by steroids, immunosuppressants (thiopurines, methotrexate), biologicals (anti-TNFα, anti-adhesion molecule, anti-IL12/23). The therapeutic goal is not just to relieve IBD-related symptoms but also to favor mucosal healing because it has been involved in a superior long-term prognosis including a lower surgical risk, hospitalizations, and need for systemic steroids (136). Anti-TNFα therapies have changed the prognostic of IBD but 10%-40% of patients lose response within 12 months (137) and a further 10–20% annually thereafter (138). In addition, these treatments are not devoid of side effects (139) and adherence to medications is a challenge in IBD patients (140). Surgery for IBD occurs for 70% of CD patients and 35% of UC patients (141). Surgical operation is performed in case of failure of medical treatment or complications and patients are re-operated because surgery, but also medical treatment, is not curative but only suspensive. The diagnosis of IBD is often done late at a time where lesions are evolved such as stenosis, fistula, abscesses in CD, and more refractory to medical treatment. Consequently, targeting IBD early when the disease is purely inflammatory is of interest. These patients have also a risk of recto-colonic cancer due to chronic inflammation and mucosal healing is presently a gold standard in the treatment of IBD.

Experimental

The VN anti-inflammatory activity potentiating the CAP has been reported in experimental colitis (142, 143), after vagotomy (142), VNS (144, 145), and peripheral or central injection of AChesterase inhibitors (146). Its anti-inflammatory role goes through a macrophage-dependent mechanism involving nicotinic receptors. However, other counter-inflammatory mechanisms play also a role when vagal integrity is compromised and does not play its protective role (147).

Classically, low frequency (5–10 Hz) VNS is known to stimulate vagal efferents, i.e., the CAP. However, we have shown in experimental conditions that even at low frequency stimulation vagal afferents are also activated in anesthetized rats under VNS in an fMRI study using dynamic causal modeling to estimate neuronal connectivity (148). We have also reported that long-term low

frequency (10 Hz) VNS was able to induce modifications of the electroencephalogram in a CD patient under VNS (149). In fact, in the neuroanatomic context of the pathways that are involved in the anti-inflammatory role of the VN both stimulation of vagal afferent and efferent fibers is of interest.

Using VNS in a rat model of TNBS colitis classically used for CD, we have shown that low frequency (5 Hz) chronic VNS performed for five consecutive days with parameters classically used for epilepsy improved colitis (144). Indeed, a multiparametric index of colitis taking into account clinical, biological, macroscopic and histological damage, as well as pro-inflammatory cytokines, was improved in rats under VNS. We observed that VNS was more efficient on the area of lesion with less inflammation located immediately above the principal inflammatory lesion. In the same experimental colitis model, Sun et al. (145), have also evaluated the chronic VNS effect but with a higher frequency stimulation (20 Hz) on colonic inflammation using clinical, histological, and biochemical parameters. They also recorded HRV in rats with colitis under VNS. They observed a significant decrease of colitis under VNS and IL-6 and TNF-α cytokines, and show an improvement of the sympatho-vagal balance. Very recently, in a similar approach, Jin et al (150), using the same model of TNBS colitis, showed that chronic VNS improved colonic inflammation by inhibiting pro-inflammatory cytokines *via* the autonomic mechanism; addition of non-invasive electroacupuncture to VNS enhanced the anti-inflammatory effect of VNS.

Clinical

Until recently, only few data were available concerning the anti-inflammatory role of the VN in IBD. However, recording of vagal tone and the sympatho-vagal balance using HRV, a reliable non-invasive tool that quantifies sympathetic and parasympathetic activities, allows such an approach. The risk for developing a chronic disease is associated to a dysregulated ANS with a decreased vagal tone. In the context of brain-viscera interaction, HRV monitoring is an important tool which allows the sensing of vagal tone and its impairment and, hence, the CAP deficiency. HRV monitoring is a biomarker which predicts the prognosis of several chronic inflammatory diseases (151). As we know that a decrease in vagal tone induces a reduction in HRV. We have shown in IBD patients a correlation between vagal tone and emotional adjustment (low negative emotions vs high negative emotions) and the way of how patients coped with their disease. A positive coping profile was associated with a low vagal tone in CD and with a high vagal tone in UC (3). Consequently, it is important to separate IBD patients according to the disease (CD vs UC) as well as the importance of psychological factors on vagal tone. In addition, recent data have shown that an autonomic dysfunction precedes the development of RA (4). We have also reported that CD patients with a low resting vagal tone presented higher blood TNFα and salivary cortisol levels than patients with high vagal tone (135). A low vagal tone is thus associated with a pro-inflammatory state. In addition, based on the fact that stress inhibits the VN and thus favors a pro-inflammatory state, this may explain, at least in part, that stress could favor a relapse in IBD patients. In this context, monitoring resting vagal tone over time could be useful (a) for predicting vulnerable state, (b) for proposing adapted enforcement therapy such as complementary medicine, known to stimulate the VN, pharmacological manipulation of the CAP, or VNS to restore a normal vagal tone, and (c) for a follow-up of the therapy efficacy on the parasympathetic system.

In a translational approach in CD patients, we have performed a pilot VNS study where 7 patients with active ileo-colonic CD where implanted with a VNS device. Only two patients out of seven were on treatment (Azathioprine) on inclusion. We have recorded clinical (Crohn's disease activity index, CDAI), biological (CRP, fecal calprotectin), endoscopic (Crohn's disease endoscopic index of severity, CDEIS) markers of activity during a 6 months of follow-up. The first implanted patient was on April 2012 and the 7th patient on November 2014. All the patients entered in a follow-up study. VNS induced deep remission in five of the seven patients. Two patients were taken off the study after a 3 months VNS and switched to infliximab and azathioprine, one was operated (ileo-cecal resection). These two patients had the highest CDAI, CRP and CDEIS on inclusion which suggests that VNS, as a slow-acting therapy, is more indicated in moderate CD. All the patients have kept the device in place with the duty cycle still running, except one of the two patients removed from the study who have a low intensity of stimulation (0.5 mA). VNS was well tolerated with the classical minor side effect represented essentially by hoarseness. We did not have any problem of infection either local or systemic and no VNS device was removed. The data on the first seven patients after a 6-month follow-up were reported for the first time recently (152). VNS could also be used to maintain remission induced by drugs. Surgery is used to cure CD lesions and VNS as a slow-acting therapy could be an interesting tool to prevent postoperative recurrence of CD.

Irritable Bowel Syndrome

Abdominal pain, bloating and altered bowel habits without any organic cause with a higher prevalence in women (153) are the main characteristics of IBS. IBS prevalence goes from 10 to 15% in industrialized countries (154) and represents up to 12% primary care doctors and 28% gastroenterologist medical visits (155). Significant impairment in quality of life, time off work, and significant increase in health care costs are the principal consequences of IBS. Extra-intestinal manifestations such as headache, arthralgia, urinary problems, insomnia, and fatigue are classically reported by the patients in association with digestive symptoms. Fibromyalgia, frequently associated with IBS, worsens digestive symptoms (156). Psychological factors as anxiety or major depression, are often observed in IBS patients (up to 50%) (157). Stress has a major role in the pathophysiology of IBS (132). In particular, early life trauma such as a history of emotional, sexual, or physical abuse is reported in 30–50% of patients (158) and symptoms are often triggered by stress. Intestinal distension–induced visceral hypersensitivity and characterized by lower pain thresholds is often observed in IBS patients and is a classical marker of the disease (159). Mechanisms of this visceral hypersensitivity seem to be explained by a low-grade inflammation in the GI

tract (that could favor modifications of neuronal plasticity) (160) and by a mast cells sensitization of intestinal afferent terminals (161). Bacterial gastroenteritis is associated with 4–30% of post-infectious IBS (162). However, anxiety, high levels of perceived stress, somatization and negative illness beliefs at the time of infection were also predictors of post-infectious IBS (163), arguing for a cognitive-behavioral model of IBS. IBS has been compared to an IBD "a minima" since an increased number of gut mucosal T-lymphocytes and mast cells as well as an increased of blood level pro-inflammatory cytokines (IL-10 and IL-12, suggesting Th1 polarization) have been described (164). Globally, IBS is described as a biopsychosocial model due to a blunted brain–gut axis consistent with an up-regulation in neural processing between gut and brain. Patients are hypervigilant toward their symptoms explaining visceral hypersensitivity. Central sensory processes are modified in IBS patients (165) and this is assimilated to a central sensitization syndrome (166, 167). Dysautonomia, a marker of brain-gut dysfunction, has been described with a high sympathetic and a low parasympathetic tone, irrespective to the positive or negative affective adjustment (3). Because of the multifactorial pathophysiology of IBS, its medical treatment is disappointing and essentially based to alleviate symptoms. Psychotherapy, like cognitive-behavioral therapy and complementary medicine like hypnosis, are known to improve vagal tone (90, 168, 169), and could be of interest in the treatment of IBS symptoms.

From a pathophysiological point of view, targeting both the GI tract and the central nervous system through the VN is of interest in IBS. Based on its peripheral anti-inflammatory action through the CAP and on its central effect, as antidepressive, VNS would be of major interest in IBS treatment. In addition, the VN is involved in the control of pain and VNS has been shown to modify central pain processing. Indeed, in visceral pain models in rats, VNS has been shown to increase the pain threshold (170) and to modulate visceral pain-related affective memory (171). Modification of pain by VNS has also been reported in epileptic patients certainly by modulating peripheral nociceptor function (172). Deep breathing increases cardiac vagal tone and prevents the development of acid-induced esophageal hypersensitivity in healthy volunteers; this effect was abolished by atropine (173). Somatic pain thresholds are increased in healthy volunteers with ta-VNS (174). VNS activates vagal afferents that project to brain nuclei involved in the descending inhibitory modulation of pain (175).

Presently, there is no published data on the treatment of IBS by VNS although two studies using n-VNS are registered in ClinicalTrial.gov. The first study has been set up by ElectroCore LLC, with a new n-device called GammaCore. This randomized, single center, double-blind, parallel, sham-controlled pilot study relates on the treatment of symptoms caused by functional dyspepsia or IBS (ClinicalTrials.gov Identifier: NCT02388269). Although completed, no results have been still posted. The second study, still recruiting, evaluates the effect of a 6-month transcutaneous VNS on intestinal and systemic inflammation, intestinal transit time mucosal permeability, and quality of life in IBS patients (ClinicalTrials.gov Identifier: NCT02420158). Ten IBS women, aged between 18 and 60 years, will be included.

Postoperative Ileus

Abdominal surgery induces POI whatever the localization of surgery site. POI is defined by a delayed gastric emptying and a prolonged intestinal transit (176). Stomach and small intestine functions turn back to normality within 24–48 h while the colon takes generally more time (up to 72 h). The recovery of GI motility can take longer hospitalization times and thus higher healthcare costs. The cost of this postoperative complication has been estimated at US$1 billion/year in the US (176). Sympatho-adrenergic and vagal nonadrenergic noncholinergic inhibitory efferent pathways play a role in the POI mechanisms while capsaicin-sensitive neurons are implicated in the afferent pathway of the reflex (177). Supra-spinal brain nuclei have also been implicated in POI, in particular, specific hypothalamic and pontine-medullary neurons involved in the autonomic regulation of GI function (178). A role for CRF in the PVH is evoked since CRF is a key mediator in the stress effect on the GI tract. Indeed, stress is well known to inhibit gastric emptying (179) as shown by the intracerebroventricular injection of a-helical CRF-(9–41), a CRF antagonist, which reduces the delay of gastric emptying under stress conditions (180). This effect is CRF1 receptor-dependent. More recently, a peripheral pathway, involving the CAP, has been described in the mechanism of POI. Indeed, abdominal surgery induces inflammation of the muscularis propria (181) and activation of resident macrophages which release TNFα. Depletion and inactivation of the muscularis macrophage network prevents POI. Systemic administration of selective nACh agonists as well as VNS reduces the inflammatory response to manipulation of the intestine during surgery (81). This anti-inflammatory effect, mediated by a reduction in macrophage activation and cytokine production is driven by the CAP (48). Gum chewing reduces POI by stimulating vagal activity (182). Targeting the CAP could thus improve POI by its anti-inflammatory action and VNS could therefore be a potential treatment to prevent POI.

In a mouse model of intestinal manipulation, de Jonge et al. (38) have shown that 5 min of cervical VNS prior to abdominal surgery improved GI transit through alpha7 subunit-mediated Jak2-STAT3 activation in intestinal macrophages, indicating that VNS may represent a new therapeutic approach to shorten POI. Stakenborg et al. (183) have recently explored the therapeutic potential of VNS in patients undergoing abdominal surgery for colo-rectal cancer, randomized to sham stimulation ($n = 5$), 5 Hz stimulation ($n = 6$), or 20 Hz stimulation ($n = 7$) group. They performed 1 ms and 2.5 mA during 2 min of VNS at the beginning and at the end of the surgery. They showed that abdominal VNS significantly reduced LPS-induced IL8 and IL6 production by whole blood in patients. In the same study, they showed that abdominal VNS was as potent as that of cervical VNS in a murine model of POI.

CONCLUSION

Through the HPA axis and the CAP, the VN exerts an anti-inflammatory action. There is also an anti-inflammatory vago-sympathetic pathway where the VN and the sympathetic system (i.e., the splenic nerve) act synergistically. This anti-inflammatory effect involves both vagal afferent and efferent fibers. Targeting the

VN opens new therapeutic avenues in GI inflammatory diseases such as IBD, POI, IBS, and other TNFα-mediated diseases such as RA or psoriasis. Among these therapeutic approaches, VNS, either invasive or non-invasive, appears as an interesting tool with no major side effects in the era of Bioelectronic Medicine (184). Patients with chronic diseases are open to such a non-drug therapy because they are more and more reluctant to conventional therapies in particular because of their side effects and the need of chronic use of these treatments.

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Neuroimmune Interactions in Inflammation and Acute Kidney Injury

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Inflammation contributes to the pathogenesis of a wide variety of disorders including kidney diseases. Recent advances have shown that neural pathways are able to regulate immunity and inflammation. The cholinergic anti-inflammatory pathway (CAP) is a well-studied neural circuit involving the vagus nerve that is thought to contribute to the response to inflammatory disorders. Expression of receptors for neurotransmitters is found in some immune cells, including β2 adrenergic receptors on CD4 T cells and alpha 7 subunit of the nicotinic acetylcholine (ACh) receptor on macrophages. Once nerves are activated, neurotransmitters such as norepinephrine and ACh are released at nerve terminals, and the neurotransmitters can activate immune cells located in close proximity to the nerve terminals. Thus, vagus nerve stimulation induces activation of immune cells, leading to an anti-inflammatory response. Recent studies demonstrate a non-pharmacological organ protective effect of electrical nerve stimulation, pulsed ultrasound treatment, or optogenetic C1 neuron activation. These modalities are thought to activate the CAP and attenuate inflammation. In this review, we will focus on the current understanding of the mechanisms regarding neuroimmune interactions with a particular focus on inflammation associated with kidney disease.

Keywords: neuroimmunomodulation, immunity, inflammation, vagus nerve, kidney injury

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INTRODUCTION

Recent advances have shown that the communication between the nervous and immune systems can regulate immune function and inflammation (1). The link between the nervous and immune systems depends in part on neural reflexes involving various immune cells. Receptors for neurotransmitters such as acetylcholine (ACh) and norepinephrine (NE) are found on macrophages, dendritic cells, T cells, and B cells (2). These immune cells synthesize and release neurotransmitters and respond to neurotransmitters released from nearby nerve terminals and other immune cells (3, 4). This link between the nervous and immune systems permits rapid homeostatic responses to inflammation and is poised to mediate protection from organ injury. This review will summarize our understanding of the molecular mechanisms that underlie the neural control of inflammation in experimental models of inflammation, mainly focusing on acute kidney injury (AKI) as well as in early studies in human diseases.

CHOLINERGIC ANTI-INFLAMMATORY PATHWAY (CAP)

A well-studied neuroimmune pathway is the CAP, the efferent limb of the "inflammatory reflex pathway" mediated through the vagus nerve. This concept has been described by Tracey et al. using

a lipopolysaccharide (LPS) model of inflammation (1, 5–8). Peripheral vagal afferent neurons express receptors in proximity to immune cells and detect bacterial products (pathogen-associated molecular patterns), proinflammatory cytokines, immunoglobulins and ATP through receptors (9–16). Following detection of these inflammatory molecules, receptors transduce signals from the immune cells and injured tissue and transmit them to the brainstem nucleus tractus solitarius (8, 17). Through uncertain mechanisms vagus efferent nerve is activated and the inflammatory reflex controls peripheral cytokine levels and inflammation.

Mononuclear phagocytes and CD4 T cells are key cellular components that interact to mediate the anti-inflammatory response following activation of CAP. Tracey et al. initially found that direct electrical stimulation of efferent vagus nerve significantly decreased the amount of tumor necrosis factor alpha (TNF-α) in the serum induced by LPS (8). The effect was abolished in mice deficient in the alpha 7 subunit of the nicotinic acetylcholine receptor (α7nAChRKO). α7nAChR is predominantly expressed in neuronal tissues, but Tracey et al. provided histological and functional evidence for α7nAChR on macrophages (11). LPS-induced TNF-α production in peritoneal macrophages was suppressed by ACh or nicotine, an agonist for the nicotinic receptor, and its suppression was abolished in peritoneal macrophages derived from α7nAChRKO mice (18). Downstream of α7nAChR, inhibition of the nuclear translocation of NF-κB (19) and activation of the JAK2–STAT3 pathway (20) ultimately reduces the production of inflammatory mediators.

CD4 T cells importantly contribute to the anti-inflammatory response following activation of CAP. Vida et al. revealed a role for β2 adrenergic receptors on CD4 T cells (21). They showed that the β2 antagonist butoxamine efficiently reversed the TNF-α suppression induced by vagus nerve stimulation (VNS). Furthermore, the anti-inflammatory effect of VNS was lost in β2 adrenergic receptor-deficient mice, but the VNS-induced effect was rescued by the transfer of $CD4^+$ $CD25^-$ cells (non-T regulatory cells) and not $CD4^+$ $CD25^+$ cells from WT mice (21). These results provide molecular and pharmacological evidence for the role of $CD4^+$ cells in contributing to the anti-inflammatory effect of CAP.

In addition to the importance of macrophages and CD4 T cells, it became evident that the spleen was a crucial organ in the CAP (22). Huston et al. revealed that the spleen is the major source of TNF-α after LPS challenge, and VNS-induced inhibition of systemic TNF-α production was abolished in splenectomized animals (22).

The splenic nerve, a component of the sympathetic (adrenergic) nervous system, releases NE, whereas the vagus nerve, a component of the parasympathetic (cholinergic) nervous system, releases ACh at nerve terminals upon activation. In response to VNS, ACh in the spleen (3) and NE in plasma (21) are upregulated. Rodent spleen receives noradrenergic fibers from the splenic sympathetic nerves, but little or no direct (cholinergic) innervation from the vagus was found in the spleen (23, 24). Rosas-Ballina et al. elegantly showed that spleen $CD4^+$ $CD44^{high}$ $CD62L^{low}$ cells, which express choline acetyltransferase (ChAT) and can release ACh, are the source of ACh in spleen during activation of CAP (3). Catecholaminergic terminals of the splenic nerve in the white pulp of the spleen are in close proximity to lymphocytes, including ChAT-positive T cells, thus permitting functional coupling. The anti-inflammatory effects of VNS are abolished in nude mice, which do not have functional T cells, however reconstituting T cells through adoptive transfer of ACh-synthesizing T lymphocytes into nude mice partially restores VNS-induced anti-inflammatory effect (3). These experiments explain how ACh levels are elevated in the spleen after VNS despite the lack of direct vagus innervation of the spleen. Still unresolved, however, is how NE is released in the spleen (or systemically) after VNS, information that would clarify the interaction between vagus (parasympathetic, cholinergic) and splenic (sympathetic, adrenergic) nerve in CAP. **Figure 1** characterizes the CAP based upon current information and **Figure 2A** summarizes the methods to activate the CAP.

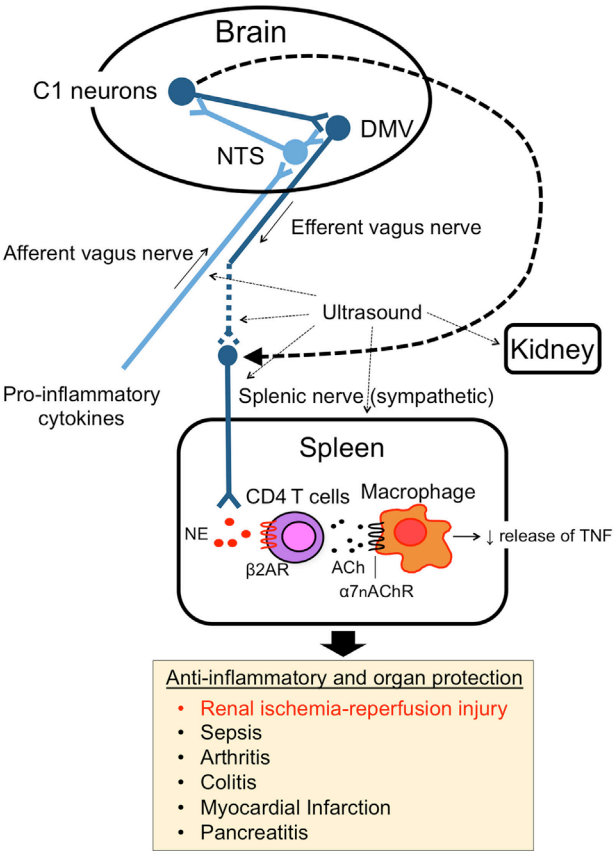

FIGURE 1 | Cholinergic anti-inflammatory pathway (CAP). The CAP links the nervous system and immune system. The activity of afferent vagus nerve fibers is stimulated by cytokines and pathogen-associated molecular pattern molecules. The signal activates efferent vagus nerve fibers through the nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus in the brain. The efferent vagus nerve (cholinergic) stimulates CD4 T cells in spleen via the splenic sympathetic (adrenergic) nerve. Release of norepinephrine binds to β2-adrenergic receptors (β2ARs) on CD4 T cells, which then elicits release of acetylcholine (ACh). ACh binding to alpha 7 nicotinic acetylcholine receptors (α7nAChRs) on macrophages produces an anti-inflammatory response, such as TNF-α suppression. Ultrasound and C1 neuron stimulation also activate the pathway and produce organ protective effects.

A

Methods to activate the CAP	Characteristics
Drugs (Nicotine, GTS-21)	Associated with off-target effects
Electrical stimulation	Electrical VNS has been used in many clinical trials
Optogenetic stimulation	Useful in identifying selective neural circuits
Pulsed ultrasound	Noninvasive; uncertain mechanism

B

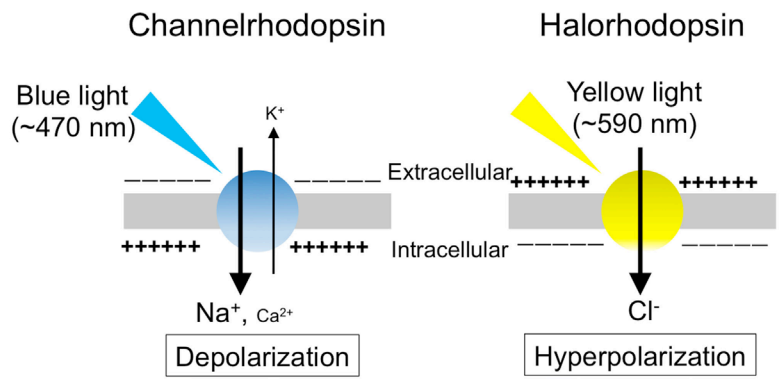

FIGURE 2 | The summary of the methods to activate the cholinergic anti-inflammatory pathway (CAP). **(A)** The list of the methods to activate the CAP. **(B)** Photosensitive ion transporters (used for optogenetic stimulation). When blue (yellow) light is applied to neurons expressing channelrhodopsin (halorhodopsin), the opsin functions as a non-selective cation channel (a chloride pump), resulting in excitation (inhibition) of the neurons.

AKI AND INFLAMMATION

Acute kidney injury is a sudden loss of kidney function that occurs within a few hours or up to a few days after an insult and is accompanied by inflammation and tissue damage. AKI causes life-threatening complications such as fluid overload, hyperkalemia, uremia, and metabolic acidosis and can also have systemic effects on other organs including brain, heart, and lung. In hospitalized patients, AKI is a common and major concern because of its high morbidity and mortality (25, 26). In addition, there is a high prevalence of AKI in emergency department patients (27). Various factors such as ischemia, drugs, toxins and infections cause AKI, and inflammation plays a major role in the pathophysiology of AKI including aseptic or sterile AKI (28, 29). The role of immune cells differ depending on the causes of AKI (30). As ischemia–reperfusion injury (IRI) is one of the major causes of AKI and has been studied most extensively, the involvement of both innate and adaptive immunity has been revealed in renal IRI (29–31) as described below.

Following renal ischemia–reperfusion, much of the inflammation occurs in the outer medulla, the part of the kidney with the lowest oxygen tension and with the greatest degree of epithelial cell necrosis. Damage-associated molecular patterns released from dying cells, adhesion molecules, and Toll-like receptors induce the recruitment and activation of various immune cells during the early injury phase (30). Recruitment of neutrophils, natural killer (NK) cells, and NK T cells occurs within several hours of tissue injury, and these cells contribute to initiation of the inflammatory cascade such as IL-1, IL-6, and TNF-α. Leukocyte infiltration into postischemic regions in the kidney is promoted by activation of the complement system in addition to enhanced proinflammatory cytokines and chemokines production (32). Renal dendritic cells increase in number in the injury site and mediate inflammation from the early to late injury phase. Inflammatory monocytes infiltrate the injury site subsequently to neutrophils infiltration and they differentiate into macrophages. In the injury phase, macrophages are polarized into proinflammatory macrophages (M1) and cause tissue damage. By contrast, anti-inflammatory macrophages (M2) predominate during the recovery phase and contribute to resolution of inflammation and tissue repair (31, 33). Tregs play important roles in tubular regeneration, promoting the repair from ischemic injury in addition to their intrinsic renal protective function (34, 35). B cells are activated in the injury phase and limit tubular regeneration in the recovery phase, leading to tubular atrophy (36).

VNS IN KIDNEY DISEASE

Inoue et al. evaluated the role of VNS in AKI (37). When VNS was performed 24 h prior to IRI, renal function and tissue morphology were preserved. Plasma TNF-α induction by IRI was also suppressed by prior VNS. Interestingly, this renal protection was observed after either afferent or efferent VNS. Vagus efferents on the opposite side were activated by stimulating vagal afferents on the left side (37). However, the kidney was still protected form IRI by left afferent vagal stimulation with the right vagal nerve blocked with local anesthetic. These findings suggest that afferent VNS and efferent VNS might protect the kidney through different mechanisms. Studies to dissect separate vagal pathways using optogenetic approaches would provide new mechanistic information.

Splenic α7nAChR-positive cells contribute to VNS-induced kidney protection. Splenocytes harvested from WT or α7nAChRKO donor mice 24 h after VNS or sham VNS treatment

were transferred into naive WT recipient mice. The recipient mice received renal IRI 24 h later. When the mice received splenocytes from VNS-treated WT mice, the recipient mice were protected from IRI. However, no protection was observed when the recipient mice obtained splenocytes from VNS-treated α7nAChRKO mice (37). The number of macrophages in the kidney increased with time after IRI, but there was no difference in number between WT and α7nAChRKO mice. On the other hand, prior VNS seems to change the polarizing phenotype of macrophages in the kidney. IRI alone induced all M1 markers and most of M2 markers we measured, but suppressed Arg1 expression (an M2 marker) in the infiltrated macrophages in the kidney. Prior VNS rescued Arg1 expression in WT mice, but not in α7nAChRKO mice (37). Thus, VNS-induced protection from kidney injury requires α7nAChR-positive splenocytes and the phenotypic change of macrophages toward M2 might be one mechanism of the protection by VNS.

Inflammation and activation of immune system are also involved in the initiation and development of other kidney diseases such as glomerulonephritis, lupus nephritis, and diabetic nephropathy (38–40). Depending on the phases and types of the kidney diseases, different immune cells, cytokines, and chemokines participate in the pathogenesis of the disease. The involvement of T cells (41) and macrophages (31, 33, 42), key components of the CAP, is reported in various kidney disorders. With rare exception, there are no reports that VNS was applied to the other kidney diseases. VNS was applied to brain dead donors in a model of kidney transplantation (43). VNS stimulation of brain dead donors led to an improvement in transplanted kidney histology and renal function at 16 weeks posttransplantation when compared to unstimulated group. These preclinical data undergird the use of VNS in clinical trials of kidney transplantation. There are limited studies in kidney diseases; however, based on the importance of inflammation and immune responses in these disorders, we believe that many kidney diseases may serve as important therapeutic targets of VNS.

ULTRASOUND (US) PROTECTS FROM AKI THROUGH THE CAP

Gigliotti et al. found that prior US application (1 s every 6 s for 2 min on the both sides of kidneys including spleen, mechanical index of 1.2) suppressed systemic and local (renal) inflammation such as IL-6 and TNF-α, and attenuated AKI (44, 45). Experiments in splenectomized mice revealed that the spleen is required for US-induced renal protection (44). US-induced protection was also observed in a cecal ligation and puncture-induced sepsis model (45). Prior US treatment did not protect the kidney in Rag1-deficient mice (Rag1KO) that lack T and B lymphocytes. However, when the Rag1KO were reconstituted with CD4 T cells 10 days prior to US treatment, the protective effect of US was restored (44). In addition, adoptive transfer of splenocytes from US-treated mice 24 h prior to IRI confers protection from IRI (45). These data indicate that CD4 T cells in the spleen are important for US-mediated renal protection.

To investigate whether the protective effects of US were mediated through α7nAChR signaling in CAP, α7nAChRKO mice were used. In α7nAChRKO mice, treatment with US before IRI did not provide protection (44). Bone marrow chimera experiments further revealed the importance of α7nAChRs on hematopoietic cells in US-related organ protection (45). Taken together, these findings suggest that US protects the kidney from AKI through activation of the CAP.

VNS AND OTHER NEUROIMMUNE REFLEXES IN VARIOUS DISORDERS

Following initial studies on LPS-mediated inflammation, anti-inflammatory effects of VNS in a wide variety disorders such as arthritis (46), colitis (47), ileus (47), pancreatitis (48), heart disease (49), diabetes (50), and hypertension (51) were confirmed. In contrast to the spleen, the vagus efferent fibers innervate the intestinal wall and directly synapse with postganglionic neurons in the enteric nervous system (52). VNS improves colitis (53) and ileus (54), and vagotomy aggravates these conditions (55). The vagus nerve does not directly innervate the joints; however, VNS attenuated limb inflammation following acute carrageenan-induced arthritis, and vagotomy resulted in increased acute inflammation as evidenced by severe edema (56). Chronic stimulation of vagus nerve, using a method called vagus nerve suspension, improved arthritic scores 3 months after the second immunization in a collagen-induced arthritis model (57). In addition, chronic electrical stimulation of vagus nerve (once daily from day 9 to day 15 for 60 s), using an implanted device with vagus nerve cuff electrodes, reduced the severity of collagen-induced arthritis in rats at day 16 (58).

On the other hand, there are many reports implying the existence of different reflexes and pathways other than the classical CAP. Sciatic nerve activation with electroacupuncture suppresses systemic inflammation following polymicrobial peritonitis through activation of the vagus nerve (59). Stimulation of the sciatic nerve induces vagal-mediated production of aromatic L-amino acid decarboxylase in the adrenal medulla leading to the production of dopamine thereby suppressing systemic inflammation through dopamine type 1 (D1) receptors. Recent studies revealed the importance of sympathetic (adrenergic) nerves (splanchnic and splenic nerves) in a neural reflex pathway that controls inflammation (60). LPS administration strongly increased splanchnic nerve and its splenic branch activity in addition to systemic TNF-α production. LPS-induced increase in plasma TNF-α was enhanced nearly five times when splanchnic nerve was cut, and vagotomy did not affect this. Plasma corticosterone levels were not affected by splanchnic nerve cut, suggesting that in this particular model the neural reflex pathway controlling inflammation was independent of corticosteroids and the vagus nerve. ACh-producing B lymphocytes control local neutrophil recruitment to the peritoneum in response to endotoxin (61). Finally, recent studies suggest a role of β2-adrenergic receptors on the muscularis macrophages, localized deeper in the gut wall (62). Upon luminal bacterial infection, sympathetic (adrenergic) neurons innervating the gut are activated, then muscularis macrophages further enhance tissue-protective programs through β2-adrenergic receptors on the macrophages (62).

OPTOGENETIC APPROACHES TO DEFINING THE MECHANISM OF THE CAP

Optogenetics is a technique involving the use of light to control the activity of neurons that have been genetically modified to express photosensitive ion transporters (**Figure 2B**) (63). The discovery of channelrhodopsin-1 (64) and -2 (65) (ChR1 and ChR2) in 2002 and 2003, respectively, marked the genesis of optogenetics. These unique opsins are found in the "eye spot" of *Chlamydomonas reinhardtii*. When blue light (~470 nm) is applied to ChR2, the channel opens and functions as a non-selective cation channel. Only 2 years after the discovery of ChR2, investigators introduced it in mammalian neurons, which were then successfully stimulated using blue light (66).

The utilization of halorhodopsin, an inhibitory channel, gave further impetus to studies using optogenetics (67). When yellow light (~590 nm) is applied to neurons expressing halorhodopsin, the pump allows chloride ions to enter the cells, thereby resulting in hyperpolarization and inhibition of the neurons. In addition, by using the Cre-Lox system, these excitatory or inhibitory proteins can be introduced into specific cell types. Thus, optogenetics enables targeted excitation or inhibition, conferring cellular or projection specificity, which is not attainable through electrical stimulation. Owing to its significant impact on studies in neuroscience, this technique was selected as the "Method of the Year" by the Nature publishing group in 2010 (68).

Because of the many advantages optogenetics provides, this technique is especially useful for revealing the mechanism of the CAP, although it has been underutilized for this purpose thus far. For example, vagal afferent sensory neurons have been divided into several subgroups based on specific markers, and optogenetic stimulation of each subgroup modulated the function of the lung, heart, and gastrointestinal tract in different ways. Selective activation of P2ry1-positive and Npy2r-positive neurons in vagal afferents resulted in apnea and rapid/shallow breathing, respectively (69). It was also reported that Gpr65-positive neurons innervating intestinal villi detect nutrients and regulate gut motility, while Glp1r-positive neurons sense stomach and intestine stretch (70). These findings suggest that nerves in the CAP such as vagus nerve and splenic nerve can be divided into several subgroups and each subgroup might have different roles in the CAP and that optogenetics has the high potential to facilitate elucidation of the selective neural circuits of the CAP.

C1 NEURONS AND RESTRAINT STRESS IN THE CAP

By using optogenetics, Abe et al. revealed a new role of C1 neurons in activating the CAP (71). C1 neurons, which reside in the medulla oblongata and innervate the dorsal motor nucleus of the vagus nerve, sympathetic (adrenergic) efferents, the paraventricular nucleus of the hypothalamus, and other brainstem regions, mediate adaptive autonomic responses to several stressors, such as hypotension, hypoxia, and LPS (72). C1 neurons stimulation by laser protects mouse kidneys from IRI. The spleen, β2-adrenergic receptors, and α7nAChRs are necessary for the protective effect, thereby suggesting that the CAP is involved in the protective effect of C1 neuron stimulation. Interestingly, restraint stress for 10 min also protects the kidney from IRI. The protective effect is mediated by C1 neurons and was abolished by ganglionic blockade but not affected by subdiaphragmatic vagotomy or by corticosterone receptor blockade. These findings suggest that C1 neurons activate the CAP not through a vagal, but through a sympathetic (adrenergic) route.

VAGUS NERVE STIMULATOR

More than 100,000 vagus nerve stimulators have been implanted for medically refractory epilepsy and for treatment-resistant depression and the device has been well tolerated (73). Although transvenous VNS failed to suppress systemic inflammation in healthy subjects (74), pilot studies have showed that implanted vagus nerve stimulators are effective in rheumatoid arthritis (75) and Crohn's disease (76) in human. In 17 patients with rheumatoid arthritis, including those in early and late stages of the disease, symptoms improved significantly during the period of VNS (75). Biological parameters, including endoscopic index of severity, improved in seven patients with Crohn's disease and treated with VNS in a 6-month follow-up (76). In addition, many other clinical trials on a wide variety of disorders such as heart failure, hypertension, inflammation, and diabetes are ongoing. Recently two non-invasive external devices have been developed to stimulate the vagus nerve through the skin (73). Transcutaneous cervical VNS reduced the extent of tissue damage after cerebral ischemic injury in rats (77). In humans, VNS with a transcutaneous device on the neck downregulated release of inflammatory cytokines, such as IL-1β and TNF-α, in healthy subjects (78).

CONCLUSION

In summary, neuroimmune interactions play very important roles in various disorders, however the mechanisms linking these two different systems are very complex and incompletely understood. Additional studies using advanced tools such as optogenetics will lead to further molecular understanding of neuroimmunomodulatory mechanisms controlling inflammation. These studies could yield potent non-pharmacological approaches to blocking inflammation and tissue injury in systemic disease. In the future, vagus nerve stimulator or US treatment might be applied to the patients who are planned to have cardiac surgery or kidney transplantation, conditions that result from renal ischemia injury.

AUTHOR CONTRIBUTIONS

All authors participated in the writing, editing, and literature search. ST was responsible for **Figure 2** and TI was responsible for **Figure 1**.

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Loss of Sympathetic Nerves in Spleens from Patients with End Stage Sepsis

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The spleen is an important site for central regulation of immune function by noradrenergic sympathetic nerves, but little is known about this major region of neuroimmune communication in humans. Experimental studies using animal models have established that sympathetic innervation of the spleen is essential for cholinergic anti-inflammatory responses evoked by vagal nerve stimulation, and clinical studies are evaluating this approach for treating inflammatory diseases. Most data on sympathetic nerves in spleen derive from rodent studies, and this work has established that remodeling of sympathetic innervation can occur during inflammation. However, little is known about the effects of sepsis on spleen innervation. Our primary goals were to (i) localize noradrenergic nerves in human spleen by immunohistochemistry for tyrosine hydroxylase (TH), a specific noradrenergic marker, (ii) determine if nerves occur in close apposition to leukocytes, and (iii) determine if splenic sympathetic innervation is altered in patients who died from end stage sepsis. Staining for vesicular acetylcholine transporter (VAChT) was done to screen for cholinergic nerves. Archived paraffin tissue blocks were used. Control samples were obtained from trauma patients or patients who died after hemorrhagic stroke. TH + nerves were associated with arteries and arterioles in all control spleens, occurring in bundles or as nerve fibers. Individual TH + nerve fibers entered the perivascular region where some appeared in close apposition to leukocytes. In marked contrast, spleens from half of the septic patients lacked TH + nerves fibers and the average abundance of TH + nerves for the septic group was only 16% of that for the control group (control: 0.272 ± 0.060% area, $n = 6$; sepsis: 0.043 ± 0.026% area, $n = 8$; $P < 0.005$). All spleens lacked cholinergic innervation. Our results provide definitive evidence for the distribution of noradrenergic nerves in normal human spleen and the first evidence for direct sympathetic innervation of leukocytes in human spleen. We also provide the first evidence for marked loss of noradrenergic nerves in patients who died from sepsis. Such nerve loss could impair neuroimmunomodulation and may not be limited to the spleen.

Keywords: noradrenergic, cholinergic, human, spleen, sepsis, innervation, remodeling

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INTRODUCTION

The central nervous system (CNS) and immune system play essential roles in maintaining homeostasis, and there is extensive evidence for crosstalk between these systems (1–5). Mediators generated during injury or inflammation stimulate sensory pathways to the brain, which responds by activating the hypothalamic–pituitary–adrenal axis and efferent autonomic neural pathways. Activation of the sympathetic nervous system (SNS) evokes well-known cardiovascular and metabolic responses aimed at addressing threats to homeostasis. As the sole source of efferent neural input to primary and secondary lymphoid tissues, the SNS also plays an essential role in CNS modulation of immune function (1, 6, 7). These effects on immune function can be indirect through modulation of blood flow or direct through stimulation of adrenergic receptors on leukocytes (1).

The spleen is the largest secondary lymphoid tissue and a major hub of innate and adaptive immune responses, making it a prime target for regulation by the SNS (1, 3, 8). Experimental studies have shown that the spleen is a major contributor to the exaggerated inflammatory response that occurs in sepsis, trauma, and burn injuries (3). Furthermore, adverse cellular and functional remodeling of the spleen contributes to immunosuppression in patients who die from sepsis and multiple organ failure (9). Accordingly, understanding neuromodulatory mechanisms that operate within the spleen has significant translational value. Neuroanatomical studies, done primarily with small animal models, have shown that sympathetic nerves enter the spleen with major vessels and distribute throughout the arterial vasculature (1, 10). Many sympathetic nerve fibers travel into the periarterial lymphatic sheath (PALS) where some occur in close apposition to T cells, B cells, and macrophages, providing a structural basis for direct neuromodulation of immune function (10–13). Diffusion of catecholamines from their release sites would also affect more leukocytes *via* so-called volume transmission (1, 2, 5, 12).

Both the spleen and its sympathetic nerves have essential roles in the cholinergic anti-inflammatory pathway (3, 4, 8). Activation of splenic sympathetic nerves by reflex mechanisms or vagal nerve stimulation triggers release of norepinephrine (NE), which stimulates β_2-adrenergic receptors on cholinergic T cells, causing release of acetylcholine (ACh). Then, ACh elicits an anti-inflammatory response mediated by α7 nicotinic ACh receptors on macrophages (3, 8). In addition to this mechanism, there is substantial evidence that catecholamines can affect the functions of macrophages, dendritic cells, T_{regs}, and B cells by stimulation of surface adrenergic receptors (1).

The translational potential for neural modulation of immune function in disease is already being tested in ongoing clinical trials of vagal nerve stimulation, which activates sympathetic input to the spleen (3). However, most studies that defined the localization of sympathetic nerves in spleen used animal models, and our knowledge regarding sympathetic innervation of adult human spleen is very sparse (14). Furthermore, experimental studies provide evidence that sympathetic innervation of the spleen can change with age and inflammatory disease (5, 15–17), but analogous human data are lacking. To address these gaps in knowledge, we used immunohistochemistry to evaluate the presence and localization of noradrenergic sympathetic nerves in sections of control spleens and spleens from patients with end stage sepsis. Additional immunostaining was done for the vesicular acetylcholine transporter (VAChT) to evaluate the presence of cholinergic nerves in the spleen.

MATERIALS AND METHODS

Human Tissue Samples

All tissue sections evaluated in this study were obtained from paraffin-embedded samples graciously provided by Dr. Richard Hotchkiss at Washington University. Many of these samples were used in prior studies of sepsis by Hotchkiss and his colleagues (9). All of these studies were approved by the Washington University or St. John's Hospital Human Research Protection Office, with informed consent, which included permission to collect tissue for research purposes, provided by a next-of-kin in accordance with the Declaration of Helsinki. Spleen samples from septic patients who died in the intensive care unit were obtained by immediate, limited autopsy performed at bedside after obtaining permission from next-of-kin. Control samples were obtained during surgical removal of the spleen to control bleeding after upper body trauma, from organ donors with permission from next-of-kin to collect tissue for research purposes, and postmortem from patients with subarachnoid hemorrhage. Clinical information provided to us and comments on hematoxylin and eosin (H&E) stained sections of spleens are summarized in **Table 1**.

Tissue Processing and Immunohistochemistry

5 μm sections of paraffin-embedded spleens and a control sample of intestine were cut using a Leica RM2135 microtome, collected on charged slides, and stained with H&E or processed for bright field immunohistochemistry using standard procedures (18, 19). Briefly, sections were deparaffinized, rehydrated, and incubated in citrate buffer (pH 6.5) for 20 min at 92°C for antigen retrieval. Sections were washed with 0.1 M phosphate-buffered saline (PBS, pH 7.3), permeabilized in PBS containing 0.4% Triton X-100 and 0.5% bovine serum albumin, and treated with 1% H_2O_2 in PBS for 15 min. After additional washing and blockade in PBS containing 5% normal goat serum, 1% BSA, and 0.4% Triton X-100, the sections were incubated with primary antibody overnight. Primary antibodies were rabbit anti-tyrosine hydroxylase (TH) (1:1,000, Pel-Freez Cat. # P40101; Rogers, AR, USA) or rabbit anti-VAChT (1:1,000, Cat. # 139103; Synaptic Systems, Göttingen, Germany). Further processing was done using a Rabbit ABC kit (Vector Laboratories), and Vector ImmPact VIP chromogen (Vector Laboratories) was used to produce a purple reaction product at sites of antigen localization. For some sections from control spleens, Vector ImmPact DAB was used as the chromogen to stain TH nerves brown, and hematoxylin was used as a counterstain to visualize nuclei. Slide-mounted sections were viewed using an Olympus BX4 microscope, and digital images were obtained using an attached Olympus Q-Color 3 digital camera and Q-Cap Pro 7 software.

TABLE 1 | Patient profiles.

Group	Gender	Age	Clinical situation	Hematoxylin and eosin histology
Controls	Female	74	Subarachnoid hemorrhage	Some white pulp (WP) loss, no germinal centers (GC)
	Female	51	CHF, COPD, subarachnoid hemorrhage	Some WP loss, rare GC
	Male	NA	Trauma	Normal
	Male	NA	Trauma	Normal
	Male	NA	Trauma	Normal
	Male	NA	Trauma	Normal, no GC
Septic	Male	63	Pancreatitis, multiple intra-abdominal abscesses, VAP, renal failure	Marked WP loss, no GC, much hemosiderin
	Female	87	S/P pelvic exenteration for cervical cancer, bacteremia w/respiratory distress, hypotension	Poorly defined WP, no GC
	Female	70	S/P femoral popliteal bypass, anemia, massive MI, vasopressors, ischemic bowel on postmortem	Decreased WP, rare GC
	Female	87	Ischemic colitis, septic about 2 days	Decreased WP, rare GC
	Male	80	Upper GI bleed and peritonitis	Marked WP loss, no GC
	Male	62	Septic w/dead gut	Marked WP loss, no GC
	Male	60	Septic w/peritonitis and obese	Some WP, empty sinusoids
	Female	NA	Influenza complicated by bacterial pneumonia	Decreased WP

Analysis of TH Nerve Fiber Abundance

We performed semi-quantitative and quantitative analyses to evaluate the abundance of TH-stained nerves fibers in spleen sections. Both analyses were performed in a blinded fashion. Semi-quantitative evaluation of TH staining was performed by a neuropathologist using the following grading scale: 0—no staining, 1—staining just around large vessels near the capsule, 2—staining around large vessels and trace staining in parenchyma including small vessels, and 3—staining around large vessels and in parenchyma.

Quantitative evaluation of TH nerve fiber abundance was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Slides were viewed with a 20× objective and scanned in a standard pattern, collecting non-overlapping images of fields containing at least one blood vessel. This process resulted in 15–45 images per section, depending on the area of the section. These images were analyzed in a blinded fashion by another individual to determine the % of each field that contained TH + nerve fibers. Resulting data are presented as % area.

Statistical Analyses

Statistical analysis of nerve abundance data was performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). Semi-quantitative data for control and sepsis groups were compared by non-parametric analysis using a two-tailed Mann–Whitney test for unpaired observations. Quantitative data were compared by using a two-tailed *t*-test for unpaired samples. A P value less than 0.05 was considered significant. Data are presented as mean ± SD.

RESULTS

Clinical and general histological details of patients from whom spleens were obtained retrospectively for examination are presented in **Table 1**. There were six control specimens: these were obtained from four trauma patients and two who died after subarachnoid hemorrhage whose spleens were obtained at autopsy. The spleens of eight patients who died with sepsis were obtained at postmortem examination. The spleens of the traumatized controls showed well-defined white pulp (WP) with numerous germinal centers (GC) and unremarkable vessels. The spleens of the controls with terminal subarachnoid hemorrhage showed well defined but less extensive WP with fewer GC. They also showed hyalinizing vascular changes that are common age-related changes at postmortem examination. The WP was greatly decreased in seven out of eight septic spleens (in the fourth it was very poorly delineated), and GC were very rare to completely lacking in all specimens. One septic spleen showed abundant hemosiderin deposition.

Sympathetic nerves were identified by immunostaining spleen sections for TH, the rate-limiting enzyme for synthesis of the noradrenergic neurotransmitter, NE. Such nerves were prominent in all control patients, with no qualitative difference between TH staining of spleens obtained at surgery compared to those obtained at autopsy. TH + nerves entered the spleen in large bundles associated with penetrating vessels (**Figure 1A**) and continued to be present in bundles of decreasing size along the arterial vasculature within the parenchyma of the spleen (**Figures 1C,D,F,H**). The splenic artery, trabecular arteries, central arteries, and smaller branches down to arterioles were surrounded by individual nerves fibers present at the adventitia-media border and in the adventitia (**Figures 1A,C–F,H–J**). Subscapular TH + nerve fibers were abundant in sections from one trauma patient, who had the most robust sympathetic innervation (**Figure 1G**). However, none of the control patients had sympathetic nerves within the capsule (**Figure 1G**). While the vast majority of sympathetic nerves in the control spleens were associated with the arterial vasculature, several TH + nerve fibers extended short distances into the PALS (**Figures 1F**, **2** and **3**). Sympathetic nerves were not present in follicles or GC (**Figures 2A,B**). Examination of perivascular regions of control human spleens at high resolution with a 100× oil objective revealed extremely close apposition of nerve fibers and varicosities with leukocytes (**Figures 2C-I** and **3**).

In sharp contradistinction to control spleens, TH + nerve bundles and fibers were lacking in four of the eight septic spleens

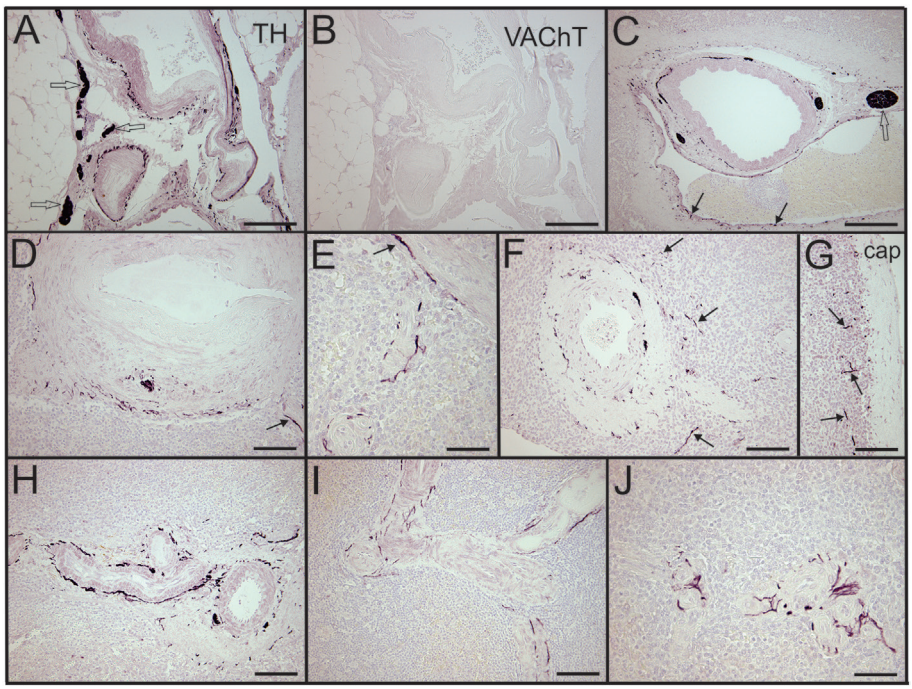

FIGURE 1 | Control human spleens had abundant tyrosine hydroxylase (TH) + sympathetic nerves associated with the vasculature and lacked cholinergic innervation. **(A,B)** Images showing noradrenergic (TH+) nerve fibers and bundles around splenic artery and branches **(A)** and absence of cholinergic (VAChT) nerves in an adjacent section **(B)** from a 74-year-old female. **(C)** Low magnification image of TH + nerve fibers and bundles around a trabecular artery in a spleen section from a male trauma patient. Arrows indicate TH + nerve fibers around an adjacent large vein. **(D,E)** TH + fibers associated with central artery and smaller branches in the same 74-year-old female. Nerve fibers are localized to adventitia and adventitia-media border. Arrow in **(D)** indicates nerve around branch of central artery shown at higher magnification in **(E)**. **(F)** Sympathetic nerves around a central artery and branch from another trauma patient. Arrows indicate some TH + nerve fibers that entered the periarterial lymphatic sheath. **(G)** Same patient had TH + nerve fibers (some indicated by arrows) in lymphatic tissue under the capsule. **(H,I)** TH + fibers around small, branching arteries in sections from first trauma patient **(H)** and from a 57-year-old female **(I)**. **(J)** Periarteriolar TH + nerve fibers in spleen section from a 74-year-old female. TH + nerve fibers extend a short distance into surrounding lymphatic tissue. Open arrows indicate some TH + nerve bundles. Scale bars: 250 μm **(A–C)**; 100 μm **(D,F–I)**; 50 μm **(E,J)**.

and greatly reduced in number in the other four (**Figure 4**). Residual TH + nerves in the latter spleens were localized primarily to larger vessels, where they occurred in much smaller bundles. Noradrenergic nerve fibers were uncommon in smaller vessels and seldom extended into perivascular regions of the septic spleens. Abnormal axonal profiles were identified among the residual TH + nerve fibers in spleens from septic patients (**Figures 4G–I**). Sympathetic nerve abundance in spleens from control and septic patients was evaluated and compared semi-quantitatively using an arbitrary grading scale and quantitatively by measuring area occupied by TH + neural elements. Both methods demonstrated significant decreases in TH + nerve fiber abundance in spleens from septic patients compared to controls (**Figure 5**). Quantitative evaluation of TH + nerve staining showed that the average abundance of TH + nerves in the septic group was only 16% of that for the control group (control: 0.272 ± 0.060% area, $n = 6$; sepsis: 0.043 ± 0.026% area, $n = 8$; $P < 0.005$).

We also evaluated adjacent sections of spleen for the presence of VAChT, which is a specific marker for cholinergic nerves. No VAChT + nerves fibers or bundles were detected in any of the spleen sections (**Figure 1B**), but prominent cholinergic nerve fibers were detected in a section of human intestine processed in the same experiment (**Figure 6**).

DISCUSSION

Sympathetic innervation of lymphoid tissues, such as the spleen, has been recognized for a long time (14, 20, 21), but the precise localization of noradrenergic nerve fibers within these tissues has only been revealed with modern neuroanatomical techniques over the past three decades. The vast majority of this work was performed using animal models, and data obtained from human lymphoid tissue are sparse (14). Our findings address this gap in knowledge by providing a detailed localization of noradrenergic nerve fibers in normal human spleen, and we also present evidence for sympathetic remodeling in patients with end stage sepsis. These findings are especially significant given recent advances in our understanding of functional interactions between sympathetic nerves and immune cells in the spleen.

We observed that noradrenergic nerves are abundant throughout the arterial vasculature of control human spleens but absent in the splenic capsule. These neuroanatomic findings are consistent with functional data obtained from early experimental

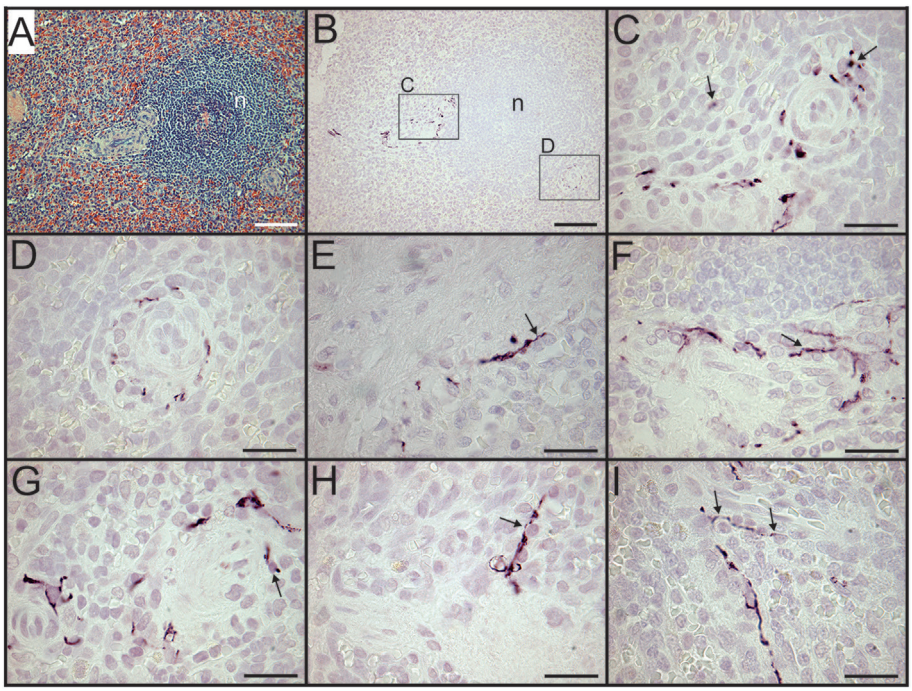

FIGURE 2 | Sympathetic nerves in perivascular lymphatic tissue of control spleens have close association with leukocytes. **(A,B)** Hematoxylin and eosin stain of small arteries associated with a splenic nodule (*n*) in section from a trauma patient **(A)** and tyrosine hydroxylase (TH) + nerve fibers around same vessels in adjacent section **(B)**. Small boxes indicate regions shown at higher magnification **(C,D)** to demonstrate close apposition of sympathetic nerves with leukocytes. **(E,F)** Close apposition of sympathetic nerves with perivascular leukocytes (arrows) in spleen sections from female patients who were 74 and 57 years old, respectively. **(G–I)** Close apposition of TH + nerve processes with perivascular leukocytes (some indicated by arrows) in spleen section from another trauma patient. Scale bars: 100 μm **(A,B)**; 25 μm **(C–I)**.

studies of isolated perfused human spleens (20, 22). The latter work showed that stimulation of postganglionic sympathetic nerves to the human spleen or close arterial injection of NE or epinephrine had only minor effects on spleen volume but caused graded vasoconstriction. This contrasts with dogs, which showed vasoconstriction and a large reduction of spleen volume due to contraction of smooth muscle in the capsule (20, 23).

Our immunohistochemical findings provide the first, high-resolution evidence for localization of sympathetic nerves in the splenic vasculature of adult humans lacking major systemic pathology. This builds on previous work, which used a fluorescence histochemical method, to visualize catecholamine-containing nerves in spleens obtained from patients with gastrointestinal pathology, primarily advanced gastric cancer (14). The latter study also reported the presence of cholinergic nerves in the human spleen based on staining for acetylcholinesterase, which is now known to be a non-specific marker that also labels some non-cholinergic nerves. Our staining for the VAChT, a selective cholinergic marker and protein required for cholinergic neurotransmission, did not detect cholinergic nerves in the spleen but did show parasympathetic nerves in a control sample from human intestines, which was processed with the spleen sections. Thus, our findings for human spleen support the conclusion, based on work with other species, that the spleen lacks cholinergic parasympathetic innervation (1, 3, 6).

While a vast majority of sympathetic nerves in the human spleen were associated with the vasculature, we observed several smooth and varicose TH + nerve fibers that entered periarterial regions of control spleens where they occurred in close proximity to leukocytes. To the best of our knowledge, this is the first demonstration of TH + nerves within leukocyte regions of the human spleen. We did not examine the phenotype of leukocytes that occurred in close apposition to TH + nerves in the human spleen, but other investigators identified T cells, B cells, and macrophages associated with noradrenergic nerve fibers in rodent spleens (10–13). It is important to note that most of these nerve fibers traveled relatively short distance into the parenchyma of the spleen. This pattern differs from results for rats, mice, and rabbits, which have dense noradrenergic innervation throughout the white pulp (24). Ultrastructural studies of rat spleen have demonstrated that some leukocyte and axonal membranes are separated by as little at 6 nm at points of close apposition (10). However, the abundance of such "synapse-like" structures was not reported, and this mode of transmission would be atypical for the autonomic nervous system where varicosities are separated from effector tissues by gaps ranging from about 20 nm to about 2 μm (25). Direct communication between noradrenergic nerve fibers and cholinergic T cells in the spleen was suggested to play a mechanistic role in the vagal anti-inflammatory pathway (13). However, recent neuroanatomical studies found that less than

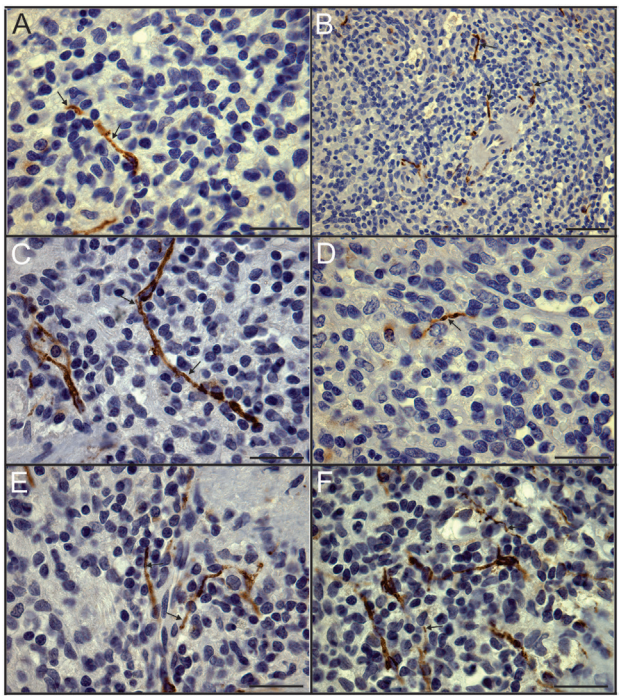

FIGURE 3 | Close association of tyrosine hydroxylase (TH) + nerves and leukocytes demonstrated in dual stained sections. **(A–F)** High magnification images of control spleen sections with TH + nerves stained brown using DAB and nuclei stained blue with hematoxylin to enhance identification of leukocytes. Arrows indicate some TH + nerves in close apposition to leukocytes. Scale bars: 25 μm **(A,C–F)**; 50 μm **(B)**.

5% of cholinergic T cells occurred within 8 μm of a TH + axon (12). These investigators proposed that communication between noradrenergic nerves and cholinergic T cells in the spleen is more likely to occur by "volume transmission," where the sphere of nerve influence is determined by diffusion of NE. Since most of the noradrenergic nerves that we identified in human spleen were localized in and close to blood vessels, diffusion of NE into surrounds PALS could provide a major mechanism for neuroimmune regulation in humans.

Much of the knowledge that we have regarding effects of adrenergic neurotransmitters on the immune system comes from *in vitro* or *ex vivo* studies of immune cells and studies of immune cell lines (1, 5). This work has shown that virtually all types of immune cell express adrenergic receptors, with the specific receptor types showing plasticity related to factors such as time, activation state, and duration of exposure to agonist. Adrenergic agonists can affect both innate and adaptive immune responses with activation of β_2-adrnergic receptors on immune cells often mediating anti-inflammatory effects and α_2 receptors mediating pro-inflammatory effects. Knowledge about specific neuronal–immune cell interactions in the spleen was sparse until the recent discovery of the cholinergic anti-inflammatory pathway. This work has established that noradrenergic nerves and cholinergic T cells in the spleen are crucial for the anti-inflammatory response to vagal nerve stimulation in endotoxemia and several other inflammatory states (3, 4, 8).

Sympathetic effects on spleen function would undoubtedly suffer during sepsis based on the deficiency of TH + nerves we detected in pathological samples. We do not know why sympathetic nerves are lost in the spleen of patients with end stage sepsis or if this change is restricted to the spleen. However, there is abundant evidence for plasticity of noradrenergic sympathetic nerves in human inflammatory diseases and animal models (5, 16, 26–28). This work suggests that nerve loss could be due to changes in the local environment that (1) decrease availability of neurotrophic factors that support sympathetic neurites, (2) increase the production of inflammatory cytokines and/or nerve repellant chemicals, and/or (3) generate toxic free radicals.

Sympathetic neurons require stimulation by nerve growth factor (NGF) for survival during development and for maintenance of sympathetic innervation throughout life (29). NGF is a target-derived neurotrophic factor that is secreted by adrenoceptive cells in organs and tissues innervated by noradrenergic nerves. Recent studies have shown that NGF is synthesized in and released by several types of immune cells including T cells, B cells, and macrophages (30–32). Since T and B cell abundance in the spleen is reduced substantially during sepsis due to apoptosis (9, 33, 34), it is reasonable to conclude that the supply of NGF to support sympathetic innervation would likewise be reduced (**Figure 7**). Previous studies identified significant loss of noradrenergic nerves and reduction of NE content in spleen and other lymphoid tissue of old F344 rats (16, 35), and several mechanisms have been proposed as causative factors in this age-dependent loss of sympathetic nerves (17, 36–38). Deficiency of neurotrophic factors ranks among these since aging-associated loss of noradrenergic nerves in the spleen and reduction of NE content occur in parallel with signs of immunosuppression (17). Aging alone is not universally associated with loss of sympathetic nerves since noradrenergic nerves are well maintained in the spleen of several strains of mice and rats (15, 39), and we observed prominent TH + nerves in spleen from a 74-year-old control female patient. Rather, it is likely that overall health and specifically health of the immune system are critical factors.

Damage from free radicals was proposed as another contributor to age-associated sympathetic nerve loss in the spleen and lymphoid tissue of rats (38, 40, 41), and free radicals are clearly generated during sepsis. Thus, reactive oxygen and nitrogen species generated in the spleen might trigger nerve loss by direct toxic effects on TH + nerves and by toxic effects on target cells, resulting in reduced production of neurotrophic factors (**Figure 7**). Several studies have shown that treatment of old rats with L-deprenyl, an agent known to increase activity of scavenger enzymes and reduce oxidative stress (42), causes partial restoration of noradrenergic nerves in the spleen and improves immune function (38, 40, 41). Part of this effect may be due to reduction of free radicals but L-deprenyl might also have neurotrophic actions (43).

Previous studies reported loss of sympathetic nerve fibers in several tissues during inflammation, an effect most thoroughly studied in synovial tissue from patients with rheumatoid arthritis (RA) and in experimental models of RA (5). Upregulation of nerve repellant factors such as semaphorin 3C and 3F in inflamed joints has been implicated in the selective loss of TH + nerves

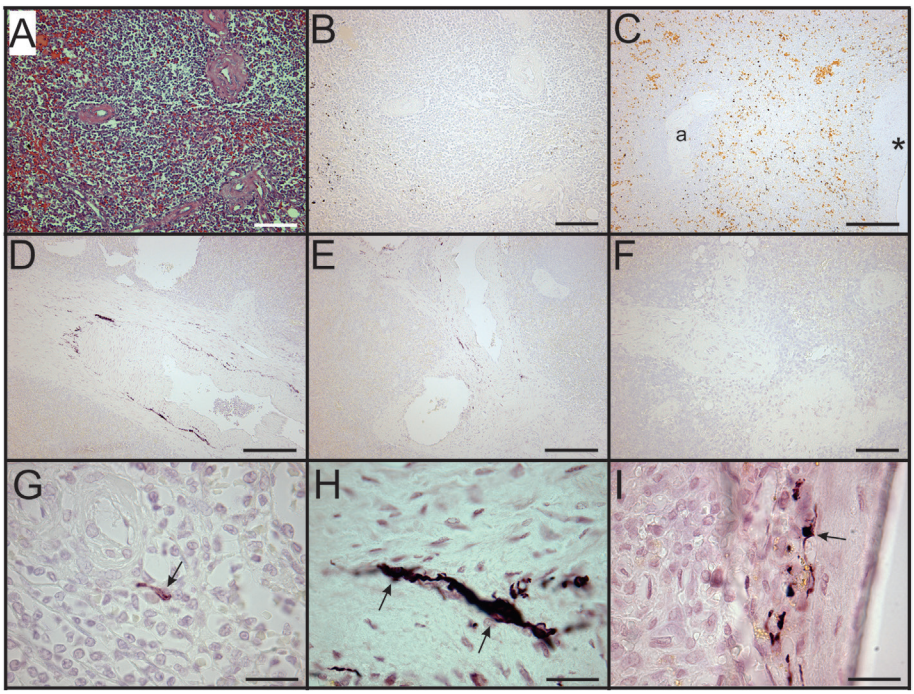

FIGURE 4 | Loss of sympathetic nerves in spleens from patients who died with sepsis. **(A,B)** Hematoxylin and eosin stain **(A)** and tyrosine hydroxylase (TH) stain **(B)** of spleen section from 63-year-old male. Note absence of TH + nerve fibers around arteries. **(C)** Absence of TH + nerves around large artery (*) and smaller artery (a) in spleen section from an 87-year-old female patient. Sections from both of these patients lacked sympathetic nerves. **(D–F)** Images showing reduced abundance of periarterial TH + nerve fibers in spleen from a 70-year-old female patient who died with sepsis. Large **(D)** and intermediate **(E)** diameter arteries show marked reduction of TH + nerve fibers compared to control patients and lacked large TH + nerve bundles. Smaller arteries **(F)** lacked TH + nerve fibers in this patient. **(G)** Solitary axon retraction ball in white pulp. **(H)** Tortuous reactive axonal profile with highly variable axonal diameter. **(I)** Axonal swelling where apparently intact axon profiles enter and leave zone of interrupted axonal transport. Scale bars: 100 μm **(A,B,F)**; 250 μm **(C–E)**; 25 μm **(G–I)**.

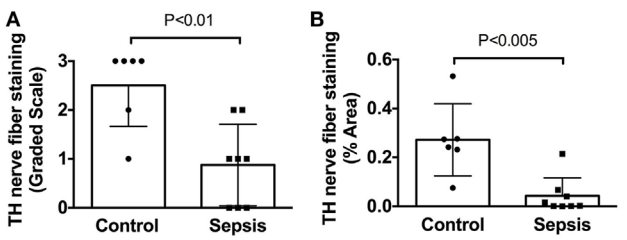

FIGURE 5 | Quantification of tyrosine hydroxylase (TH) nerve fiber staining shows a significant deficit in spleens from patients with end stage sepsis. **(A)** Semi-quantitative evaluation of TH nerve fiber abundance was performed using a grading scale ranging from 0 to 3 as described in Section "Materials and Methods." **(B)** Quantitative evaluation of TH nerve fiber abundance was performed, as described in Section "Materials and Methods," using ImageJ to measure the % area occupied by TH + nerve fibers. Both methods showed a significant reduction of TH + nerve fibers in spleen samples from septic patients compared to control patients. Data in panel **(A)** were evaluated using a two-tailed Mann–Whitney test for unpaired samples ($n = 6$ for control and $n = 8$ for septic; $P = 0.0077$). Data in panel **(B)** were evaluated using a two-tailed t-test for unpaired samples ($P = 0.0024$). Values are mean ± SD.

in RA (26). Semaphorin molecules are important axon guidance factors during development and function by activating heteromeric receptors comprising a neuropilin ligand-binding unit and a plexin signaling unit (26, 44, 45). Immunohistochemical studies have localized neuropilin-2 to sympathetic nerve fibers in synovial tissue from patients with RA and osteoarthritis (26), so expression of semaphorin receptors by sympathetic neurons and nerve fibers continues in adults. It is interesting that remodeling of sympathetic innervation has been reported in the spleen of rats with adjuvant-induced arthritis (28). In this model, TH + nerve fiber abundance is reduced in the PALS and undergoes sprouting in the red pulp. Given the inflammatory milieu present in the spleen during sepsis, it is feasible that increased synthesis and release of semaphorins might occur and contribute to sympathetic nerve loss (**Figure 7**).

Inflammatory cytokines might also contribute to sympathetic nerve loss in the spleen. Excessive production of inflammatory cytokines is a major cause of pathophysiology in patients with sepsis, and some of these cytokines [i.e., interleukin-1β and tissue necrosis factor-α (TNF-α)] have been implicated in CNS neurodegeneration (46, 47). Sympathetic neurons are known to contain receptors for several cytokines (48–52), and TNF-α in particular has actions during development to promoted apoptosis and inhibit neurite growth (49, 53). However, there is no evidence for neurotoxic effects of cytokines on adult sympathetic neurons and some can actually stimulate neurite growth (50–52). Thus, it is unlikely that cytokines contribute to the TH + nerve loss in patients with end stage sepsis.

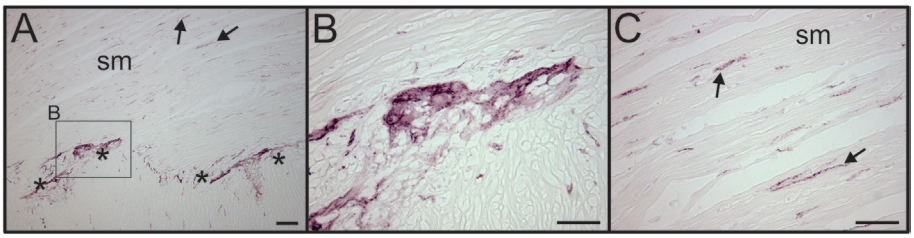

FIGURE 6 | VAChT antibody labels cholinergic nerve fibers in human intestine. **(A)** Low magnification image showing the presence of VAChT + cholinergic nerve fibers in a section of human intestine that was stained along with spleen sections. Asterisks indicate a ganglionated nerve plexus. Boxed region contains a ganglion shown at high magnification in panel **(B)**. Arrows indicates two of many VAChT nerve fibers that innervated intestinal smooth muscle (sm). **(B)** VAChT + nerve fibers surrounding an enteric ganglion. **(C)** VAChT + nerve fibers in sm region shown at higher magnification. Scale bars: 100 μm **(A)**; 50 μm **(B,C)**.

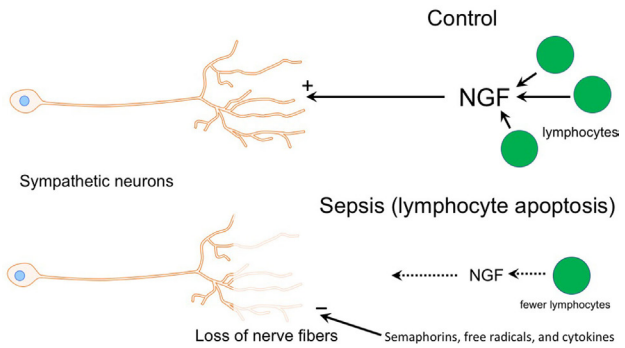

FIGURE 7 | Diagram illustrating potential mechanisms contributing to loss of sympathetic nerves in end stage sepsis. Top panel shows normal condition (control) where sympathetic innervation is maintained by nerve growth factor (NGF), which is most likely supplied by T and B cells. The bottom panel depicts changes in sepsis that could contribution to the loss of sympathetic nerves. Loss of lymphocytes in the septic spleen would decrease the supply of NGF, which is needed to maintain sympathetic nerves. Semaphorins, which are nerve repellants, could be generated in the septic spleen and cause retraction of sympathetic nerves through their interaction with the neuropilin-2/plexin A2 receptor complex located on tyrosine hydroxylase (TH) + nerve fibers. Toxic free radicals and inflammatory cytokines, generated locally during sepsis, might contribute to degeneration of TH + nerve fibers.

While this study establishes that sympathetic innervation of the spleen is reduced or lost in patients with end stage sepsis, the clinical impact of this deficit remains a matter of speculation. One possibility is that sympathetic nerve loss might favor immunosuppression, but it is also possible that altered immune phenotype contributes to nerve loss. In any event, loss of sympathetic nerves would preclude any effects that they might have to restore immune function in the spleen.

This study has some limitations that merit consideration. Sample size was small but adequate for this initial inquiry. More importantly, we had limited information on the clinical history of sepsis patients. Knowing the duration of sepsis would be useful since this parameter is likely to affect the magnitude of nerve loss. In this regard, we did note that one patient had sepsis for only a few days before dying, and that patient had the highest nerve abundance within the sepsis group. Age information was lacking for some of the trauma patients, but these individuals are generally younger than sepsis patients. Accordingly, we cannot eliminate the possibility that some loss of sympathetic innervation occurred with aging. Age-related loss of sympathetic nerves in spleen has been variable in animal studies, depending on strain (15, 39). Patients with sepsis often have multiple comorbidities, and we do not know if these affect sympathetic innervation of the spleen. Therefore, it would be important for future study to compare sympathetic innervation in of spleens from non-septic, elderly patients with and without comorbidities. Despite these limitations, this study has established a solid foundation that supports the translational potential for neuromodulation of immune function by noradrenergic nerves in the spleen. Further work is needed to define the phenotypes of splenocytes that receive noradrenergic contacts, the abundance of such contacts, adrenergic receptors expressed by different leukocyte populations, and plasticity of both nerves and receptors in disease.

In summary, we present definitive evidence for the distribution of noradrenergic nerves in normal human spleen and the first evidence for the presence of sympathetic nerves near leukocytes located in the PALS of human spleen. In addition, we provide the first evidence for marked loss of noradrenergic nerves in patients who died from sepsis. We speculate that loss of splenic noradrenergic nerves may lead to impaired neuromodulation in sepsis and this may not be limited to the spleen.

ETHICS STATEMENT

All tissue sections evaluated in this study were obtained from paraffin-embedded samples graciously provided by Dr. Richard Hotchkiss at Washington University. Many of these samples were used in prior studies of sepsis by Hotchkiss and his colleagues (9). All of these studies were approved by the Washington University or St. John's Hospital Human Research Protection Office, with informed consent, which included permission to collect tissue for research purposes, provided by a next-of-kin in accordance with the Declaration of Helsinki. Spleen samples from septic patients who died in the intensive care unit were obtained by immediate, limited autopsy performed at bedside after obtaining permission from next-of-kin. Control samples were obtained during surgical removal of the spleen to control bleeding after upper body trauma, from organ donors with permission from next-of-kin to collect

tissue for research purposes, and postmortem from patients with subarachnoid hemorrhage.

AUTHOR CONTRIBUTIONS

The study was planned by DH and DW. TB, MM, and DH performed experiments. DH and JS evaluated stained sections, and DH collected images and prepared figures. DH and JS wrote the manuscript and all authors reviewed and commented on it.

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Expression and Function of the Cholinergic System in Immune Cells

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T and B cells express most cholinergic system components—e.g., acetylcholine (ACh), choline acetyltransferase (ChAT), acetylcholinesterase, and both muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively). Using ChAT^{BAC}-eGFP transgenic mice, ChAT expression has been confirmed in T and B cells, dendritic cells, and macrophages. Moreover, T cell activation *via* T-cell receptor/CD3-mediated pathways upregulates ChAT mRNA expression and ACh synthesis, suggesting that this lymphocytic cholinergic system contributes to the regulation of immune function. Immune cells express all five mAChRs (M_1–M_5). Combined M_1/M_5 mAChR-deficient (M_1/M_5-KO) mice produce less antigen-specific antibody than wild-type (WT) mice. Furthermore, spleen cells in M_1/M_5-KO mice produce less tumor necrosis factor (TNF)-α and interleukin (IL)-6, suggesting M_1/M_5 mAChRs are involved in regulating pro-inflammatory cytokine and antibody production. Immune cells also frequently express the α2, α5, α6, α7, α9, and α10 nAChR subunits. α7 nAChR-deficient (α7-KO) mice produce more antigen-specific antibody than WT mice, and spleen cells from α7-KO mice produce more TNF-α and IL-6 than WT cells. This suggests that α7 nAChRs are involved in regulating cytokine production and thus modulate antibody production. Evidence also indicates that nicotine modulates immune responses by altering cytokine production and that α7 nAChR signaling contributes to immunomodulation through modification of T cell differentiation. Together, these findings suggest the involvement of both mAChRs and nAChRs in the regulation of immune function. The observation that vagus nerve stimulation protects mice from lethal endotoxin shock led to the notion of a cholinergic anti-inflammatory reflex pathway, and the spleen is an essential component of this anti-inflammatory reflex. Because the spleen lacks direct vagus innervation, it has been postulated that ACh synthesized by a subset of CD4$^+$ T cells relays vagal nerve signals to α7 nAChRs on splenic macrophages, which downregulates TNF-α synthesis and release, thereby modulating inflammatory responses. However, because the spleen is innervated solely by the noradrenergic splenic nerve, confirmation of an anti-inflammatory reflex pathway involving the spleen requires several more hypotheses to be addressed. We will review and discuss these issues in the context of the cholinergic system in immune cells.

Keywords: dendritic cell, lymphocyte, macrophage, mAChR, nAChR, SLURP-1

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INTRODUCTION

Acetylcholine (ACh) is one of the old neurotransmitters identified in the central and peripheral nervous systems. First synthesized in 1867 by von Baeyer, who acetylated choline using acetylchloride, ACh was left on the chemical list for several decades without exploration of its biological activity [see a review by Burgen (1)]. In 1914, however, Ewins (2) identified ACh as the active principle in ergot that exerts an inhibitory effect on the heart but a stimulatory effect on intestinal muscle. This was the first discovery of ACh in a life form. Those findings prompted Dale (3) to extensively investigate the biological activities of choline derivatives, including ACh. A little later, Loewi (4) demonstrated that the effects of autonomic nerve impulses were transmitted through peripheral release of a specific chemical stimulant in isolated frog heart preparations; this was later proved pharmacologically to be ACh (5). Dale and Dudley (6) then successfully isolated ACh from the spleens of an ox and a horse, making them the first to isolate ACh from an animal organ. On the basis of these findings, and in conjunction with the resemblance between the effects of sympathetic nerves and those of adrenaline (7), Dale (8) suggested the term "cholinergic" to describe nerves that transmit their action through release of ACh, and "adrenergic" for those who employ a substance resembling adrenaline. In 1936, The Nobel Prize in Physiology or Medicine was awarded jointly to Sir Henry H. Dale and Otto Loewi "for their discoveries relating to chemical transmission of nerve impulses." Since then, ACh has been widely recognized as a neurotransmitter.

The expression of muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) in lymphocytes and thymocytes has been known since early 1970s, based on the various functional and biochemical changes elicited by ACh and agonists such as carbachol, oxotremorine (Oxo), and nicotine in these cells [see a review by Kawashima and Fujii (9)]. Moreover, expression of mAChRs and nAChRs in lymphocytes and thymocytes was confirmed in binding studies using radiolabeled mAChR and nAChR ligands such as [^{3}H]quinuclinidyl benzilate, [^{3}H]nicotine, and [^{125}I]α-bungarotoxin (α-BTX) [see reviews in Ref. (9–12)]. At the time of its discovery, the origin of the splenic ACh isolated by Dale and Dudley (6) was left unexplored, and the findings summarized above were interpreted to show control of immune cells by the parasympathetic nervous system *via* ACh. Although the anatomy of immune system innervation has not yet been fully described, it is now generally agreed that the spleen receives innervation by sympathetic neurons but not by parasympathetic cholinergic neuron (10, 13–16). And the enigma of the origin of ACh that should act on the mAChRs and nAChRs on immune cells was ultimately solved based on the discovery of ACh in the blood and its localization to lymphocytes using a sensitive and specific radioimmunoassay for ACh (17, 18); also see reviews (9, 11, 12). Thereafter, data from a variety of investigations provided evidence that immune cells possess all the required components to constitute an independent cholinergic system, including choline acetyltransferase (ChAT, EC 2.3.2.6) and acetylcholinesterase (AChE, EC 3.1.1.7) as well as mAChRs and nAChRs [see reviews in Ref. (9, 11, 12)]. Furthermore, as reviewed by Fujii et al. (16), recent findings on the cholinergic system in immune cells suggest that ACh synthesized by immune cells plays a key role in the regulation of immune function by triggering signals that initiate and terminate cytokine production in immune cells.

In this review, we will discuss (1) the cholinergic components expressed in T and B cells, macrophages, and dendritic cells (DCs); (2) the functions of AChRs in the regulation of immune cell activity; and (3) the functions of the immune cell cholinergic system within an anti-inflammatory reflex.

CHOLINERGIC COMPONENTS EXPRESSED IN IMMUNE CELLS

We will first discuss the following major cholinergic system components: (1) ACh and ChAT, an ACh-synthesizing enzyme; (2) the ACh-degrading enzymes AChE and butyrylcholinesterase (BuChE, EC 3.1.1.8); (3) mAChRs and nAChRs; and (4) secreted lymphocyte antigen-6/urokinase-type plasminogen activator (SLURP)-1 and -2, two endogenous positive allosteric ligands for α7 and α3 nAChRs.

ACh and ChAT

In immune cells and in the central and peripheral nervous systems, ACh is synthesized from choline and acetyl coenzyme A (acetyl-CoA) by ChAT.

ACh in Immune Cells

After discovery of ACh in the peripheral blood and plasma of humans and animals, the presence of ACh in immune cells was first demonstrated in the human peripheral blood mononuclear leukocyte (MNL) fraction, which consists mainly of lymphocytes and a small monocyte fraction (18–23); also see a review (9). Later, the presence of ACh in immune cells was confirmed by detection of ACh in various human leukemic cell lines (24, 25) and rat lymphocytes, including T and B cells (26). These findings provided an explanation for the seemingly enigmatic observation of Dale and Dudley (6) that ACh was present in the spleen, though that organ is not cholinergically innervated.

In general, human leukemic T cell lines had higher ACh contents than B cell lines, prelymphoma cell lines, or a monocytic cell line (**Table 1**) (9, 25). Among rat lymphocytes, the ACh content in T cells was significantly higher than in B cells, and the ACh content in CD4$^+$ T cells was significantly higher than in CD8$^+$ T cells (26). The higher ACh content observed in rat T cells than B cells reflects the higher ChAT activity in T cells (27). Little information is available on the intracellular ACh contents in macrophages and DCs.

It is important to note that, with the molecular weight of 146 Da, ACh is small, water soluble, and both physicochemically and enzymatically fragile. Furthermore, the chemical nature of ACh is quite different from that of catecholamines and serotonin, which are able to be fixed to a tissue using paraformaldehyde. At present, no technique is available to fix ACh to the tissue. It is therefore currently impossible to detect ACh in tissues or cells using immunohistochemical or immunocytochemical techniques. Although ACh may bind to an antibody, because it is

TABLE 1 | ACh content, ChAT and CarAT activities, and ChAT mRNA expression in human leukemic cell lines.

Cell line	Cell type	ACh content pmol/10^6 cells	ChAT activity pmol/mg protein/min	CarAT activity pmol/mg protein/min	ChAT mRNA expression
CEM	T	12.6 ± 0.6	2.9 ± 0.2	22.8 ± 4.6	Positive
HSB-2	T	36.2 ± 3.5	1.4 ± 0.1	58.3 ± 15.3	Positive
Jurkat	T	8.2 ± 0.4	4.3 ± 0.8	17.2 ± 1.9	Positive
MOLT-3	T	251.5 ± 34.9	22.4 ± 3.0	53.3 ± 5.6	Positive
MOLT-4	T	38.8 ± 5.9	8.0 ± 1.0	NT	Positive
BALL-1	B	ND	0.4 ± 02	NT	Negative
Daudi	B	1.2 ± 0.1	0.1 ± 0.02	125.6 ± 44.2	Negative
NALM-6	B	0.04 ± 0.01	0.1 + 0.02	NT	Negative
REH	Pre lymphoma	0.8 ± 0.01	0.2 ± 0.03	NT	Negative
U937	Monocytic	0.02 ± 0.01	0.2 ± 0.03	NT	Negative

Values are mean ± SEM.

ND, not detectable; NT, not tested.

ChAT activity was calculated from the difference in the ACh-synthesizing activities in the presence and absence of 100 μM bromoacetylcholine. CarAT activity was calculated from the difference in the ACh-synthesizing activities in the presence and absence of 100 μM bromoacetylcamitine. Data arranged from the studies by Fujii et al. (25) and Kawashima and Fujii (9).

not anchored to its site, it will be washed away with the antibody. Nonetheless, Takahashi et al. (28) reported a successful attempt to localize ACh in the mouse gut sections using a tandem imaging mass spectrometry.

ChAT in Immune Cells

Kajiyama et al. (29) observed that a major portion of rabbit blood ACh is present in the buffy coat along with ACh-synthesizing activity. On the basis of that observation, Rinner and Schauenstein (27) confirmed the expression of ACh-synthesizing activity in rat T and B cells in the thymus, spleen, and blood, suggesting the presence of ChAT in lymphocytes.

ChAT is primarily responsible for ACh synthesis within the nervous systems of mammalian species (30). However, in peripheral tissues and non-neuronal cells, the mitochondrial enzyme carnitine acetyltransferase (CarAT, EC 2.3.1.7) also contributes to ACh synthesis along with ChAT (30, 31). ACh-synthesizing activity determined in peripheral samples using the so-called Fonnum method (32) with [^{3}H]acetyl coenzyme A and choline reflects the total activities of ChAT and CarAT. It is therefore recommended that one determines ChAT and CarAT activities in the presence of respective specific inhibitors, bromoacetylcholine and bromoacetylcarnitine. ChAT activity is proportional to the ACh content in cells of the T cell lines expressing ChAT mRNA (**Table 1**) (10, 25). However, Daudi B cells, which do not express ChAT mRNA, contained little ACh despite of a high CarAT activity. This suggests that ChAT is responsible for ACh synthesis in immune cells (10).

Expression of ChAT Enzyme Protein and mRNA

Fujii et al. (33) provided definitive evidence for the synthesis of ACh by ChAT in T cells by demonstrating expression of ChAT mRNA and the enzyme protein in MOLT-3 human leukemic T cells, using reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis (**Figure 1A**). Later, constitutive ChAT mRNA expression was detected in other human leukemic T cell lines (25), human blood CD4$^+$ T cells (**Figure 1B**) (34), rat T and B cells (24, 26), and rat MNLs isolated from the renal vasculature (35). These findings support the idea that ACh production catalyzed by ChAT is occurring in lymphocytes, including T and B cells.

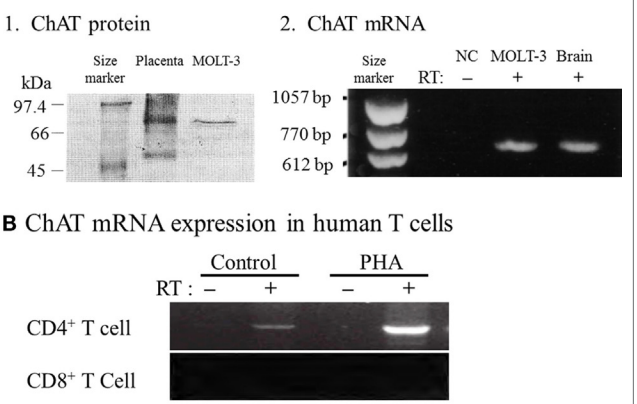

FIGURE 1 | Expression of choline acetyltransferase (ChAT) mRNA and protein in human immune cells. **(A1)** Western blot analysis of ChAT protein expression in MOLT-3 human leukemic T cells. **(A2)** Expression of ChAT mRNA detected using reverse transcription-polymerase chain reaction (RT-PCR). NC, negative control of MOLT-3 without RT. Arranged from study by Fujii et al. (33). **(B)** Expression of ChAT mRNA in human CD4$^+$ T cells and its potentiation by immunological activation with phytohemagglutinin (PHA). Note that CD8$^+$ T cells do not express ChAT mRNA, even after immunological activation. RT, reverse transcriptase. Arranged from study by Fujii et al. (34).

Reverse transcription-polymerase chain reaction revealed ChAT mRNA expression in C57BL/6J mouse spleen-derived MNLs activated with concanavaline A (ConA) and bone marrow-derived DCs activated with lipopolysaccharide (LPS) (36). However, there was no detectable expression of ChAT mRNA in these cells under resting conditions, which suggests that immunological activation is required for ChAT transcription in these cells. While no ChAT mRNA was detected in peritoneal macrophages under either resting or LPS-activated conditions, even

after an amplification protocol entailing 40 cycles (36), Koarai et al. (37) detected expression of ChAT mRNA in human lung and alveolar macrophages and monocytes using an RT-PCR protocol entailed 45 cycles, which suggests marginal ChAT mRNA expression in these cells. ChAT mRNA and protein were also detected in human mature and immature DCs using RT-PCR and immunocytochemistry (38). These findings confirm the expression of ChAT mRNA in T and B cells, DCs, and macrophages.

Expression of a Fluorescent ChAT-Reporter Protein

The recent development of ChAT^{BAC}-eGFP transgenic mice (39) and ChAT-Cre-tdTomato mice (40) provide the opportunity to detect ChAT-expressing cells using fluorescent reporter proteins. Tallini et al. (39) observed eGFP expression in a subset of lymphocytes in Peyer's patches, leading to the detection of ChAT-GFP in splenic CD4$^+$ T cells (15) and CD8$^+$ T cells and B cells (41). In addition, Gautron et al. (40) observed expression of the reporter protein in T cells within Peyer's patches in mice expressing tdTomato fluorescent protein in ChAT-expressing cells. These results confirm the earlier findings that peripheral blood CD4$^+$ and CD8$^+$ T cells and B cells express ChAT mRNA and contain ACh (26, 36).

ChAT expression in splenic DCs was confirmed using ChAT^{BAC}-eGFP mice (41). That finding along with the ChAT gene expression in DCs described above (36) suggests DCs are able to synthesize ACh using ChAT. So far, however, no additional data on ACh synthesis and release in DCs has been reported, and the physiological significance of ACh in DCs is yet to be determined.

ChAT expression was also observed in splenic macrophages using ChAT^{BAC}-eGFP transgenic mice (41). These findings are in line with those from Koarai et al. (37), who reported the expression of ChAT mRNA in human lung and alveolar macrophages and monocytes. However, Gautron et al. (40) did not find reporter expression in macrophages from gut-associated lymphoid tissue or the spleen of ChAT-Cre-tdTomato mice. As mentioned, we did not detect ChAT mRNA expression in either resting or activated mouse peritoneal macrophages (36). These findings suggest ChAT expression in macrophages may vary depending upon strain, species, tissue, cell processing procedure, and/or immunological status, or that the levels of ChAT expression are marginal in macrophages. Information on the physiological significance of ACh synthesis in macrophages is not available at present.

Regulatory Mechanisms Affecting ChAT Expression and ACh Synthesis in Immune Cells

Lymphocytes

T Cells. Fujii et al. (24) first suggested a role for ACh synthesized by T cells in the regulation of immune system function by showing that phytohemagglutinin (PHA), a T cell activator, increased both intracellular ACh content and its release into the culture medium of HSB-2 and MOLT-3 human leukemic T cells used as models of T cells. In human MNLs, PHA activates protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways *via* the T-cell receptor (TCR)/CD3 complex, resulting in specific upregulation of ChAT mRNA expression, ChAT activity, and ACh synthesis (42). However, PHA does not upregulate CarAT activity, indicating that only ChAT is linked to T cell activity (42). Upregulation of ChAT mRNA expression by T cell activation with PHA or Con A was also confirmed in rat T cells (26) and mouse spleen cells (36). These findings support the notion that immunological activation of T cells upregulates ACh synthesis, leading to modulation of immune function.

Antithymocyte globulin (ATG)-Fresenius, an immunosuppressant that binds to cell surface molecules, including CD2, CD3, CD4/CD28, CD5, CD7, CD11a (lymphocyte function-associated antigen (LFA)-1), and intercellular adhesion molecule (ICAM)-1, increases ACh release but decreases intracellular ACh content in CCRF-CEM (CEM) human leukemic T cells in the short term (6 h), and increases both ACh release and intracellular ACh content in the long term (48 h) (43). Both anti-CD11a monoclonal antibody (mAb) and ATG-F upregulates ChAT mRNA expression after 48 h of culture, suggesting activation of T cell adhesion molecules facilitates ACh synthesis (43). By contrast, acting in a manner independent of hydroxymethylglutaryl-CoA reductase inhibition, statins, including simvastatin, are able to inhibit LFA-1 (CD11a/CD18)-mediated adhesion and co-stimulation of lymphocytes, leading to immune modulation (44). Simvastatin thus abolishes anti-CD11a mAb-induced increases in ChAT mRNA expression, ACh synthesis and release in MOLT-3 cells (45). These results confirm that cell adhesion molecules such as LFA-1 contribute to the regulation of lymphocytic cholinergic activity (45, 46).

Calcium ionophores such as A23187 and ionomycin upregulate expression of ChAT mRNA and its activity, and they increase the ACh content of MOLT-3 human leukemic T cells and their culture media (46, 47). By contrast, FK-506 (tacrolimus), a calcineurin inhibitor, suppresses PHA-induced upregulation of ChAT mRNA expression and ACh synthesis, which suggests that Ca^{2+} contributes to the regulation of T cell cholinergic activity through calcineurin-mediated pathways (46).

Phorbol 12-myristate 13-acetate (PMA), a non-specific PKC activator, and dibutyryl cAMP, a protein kinase A (PKA) activator, increased ChAT activity and ACh synthesis by upregulating ChAT gene expression in MOLT-3 human leukemic T cells (46). These data provide compelling evidence that T-cell activation *via* PKC–MAPK and/or adenylate cyclase-cAMP pathways during immune responses upregulates the synthesis and release of ACh, leading to the modulation of the T cell cholinergic activity.

B Cells. *Staphylococcus aureus* Cowan I (SAC) binds to B cells and triggers a signal transduction cascade involving tyrosine kinase-mediated activation of phospholipase C (PLC) and leads to activation of PKC–MAPK pathways (48). Incubation of human circulating MNLs consisting of mainly T and B cells and a small number of monocytes with SAC for 48 h induces a significant increase of the intracellular ACh content and upregulation of ChAT mRNA expression (47). These findings suggest that stimulation of B cells also facilitates cholinergic activity *via* upregulation of ChAT mRNA expression.

Murine B cells, but not human B cells, express toll-like receptor 4 (TLR-4) (49). LPS activates murine B cells, monocytes, DCs, and macrophages by binding to a CD14/TLR-4/MD2 complex,

which leads to secretion of pro-inflammatory cytokines, nitric oxide, and eicosanoids (50). In splenic follicular B cells from ChAT^{BAC}-eGFP transgenic mice, LPS induces ChAT-GFP expression and increases ACh production (41). These results along with the aforementioned effects of SAC suggest that stimulation of TLRs on B cells activates cholinergic activity by enhancing ACh synthesis. Furthermore, ACh produced by ChAT$^+$ B cells has been shown to reduce peritoneal neutrophil recruitment during sterile endotoxemia, suggesting the role for B cell-derived ACh in the regulation of innate immunity (41).

DCs and Macrophages

As described, LPS upregulates ChAT mRNA expression in bone marrow-derived DCs, but elicits no apparent effects on ChAT mRNA expression in peritoneal macrophages from C57BL/6J mice (36). Reardon et al. (41) found that induction of ChAT expression in murine macrophages and DCs by LPS elicits MyD88-dependent signal transduction in a cell-intrinsic manner.

Activation of the Cholinergic System in Immune Cells

Lipopolysaccharide

As described, among innate immune cells, DCs and macrophages have the potential for ChAT-catalyzed ACh synthesis upon activation with LPS (36–38, 41). LPS and TLR agonists induce expression of ChAT mRNA and enzyme protein in DCs (36, 41) and macrophages (41). MyD88-dependent TLRs are involved in LPS-induced ChAT expression in DCs and macrophages (41). Because DCs and macrophages express all five M_1–M_5 mAChR subtypes (36) and various nAChR subunits (**Figure 2**) (36), these findings suggest the possibility that activation of MyD88-dependent TLRs by LPS upregulates cholinergic activity in macrophages and DCs. Furthermore, ACh synthesized in these cells should act in autocrine/paracrine fashion on their own nAChRs and mAChRs and play a role in regulation of innate immune responses by modulating cytokine production, such as tumor necrosis factor (TNF)-α and interleukin (IL)-2.

Antigen Presentation

Phytohemagglutinin and ConA upregulate ChAT expression and ACh synthesis in T cells, which indicates that antigen presentation between naïve CD4$^+$ T cells and antigen-presenting cells (APCs), including DCs and macrophages, enhances cholinergic activity in immune cells [see reviews in Ref. (11, 12, 16, 33, 47)]. Recognition by the TCR/CD3 complex of an antigen presented on major histocompatibility complex receptors on APCs triggers activation of PLCγ and Ca^{2+} release from endoplasmic reticulum (ER) in T cells (51, 52). Depletion of ER Ca^{2+} stores results in sustained Ca^{2+} influx through Ca^{2+}-release activated Ca^{2+} (CRAC) channels, leading to activation of Ca^{2+}-sensitive transcriptional factors, including nuclear factor of activated T cells, which promotes expression of cytokine genes critical for immune responses (53, 54). As described, PHA also upregulates AChE and M_5 mAChR expression along with ChAT (33, 47, 55). This suggests that ACh released from T cells and APCs (DCs and macrophages) act on their own mAChRs and nAChRs affecting autocrine/paracrine pathways, leading to modification of immune function.

The functions of M_5 mAChRs are not yet well defined at peripheral autonomic nerve effector junctions, the central nervous system, or immune cells. However, the observations that both antigen-specific IgG_1 and pro-inflammatory cytokine production are decreased in the M_1/M_5-KO mice and that M_5 mAChRs are upregulated by PHA and ConA suggest that M_5 mAChRs in immune cells are involved in positive regulation of immune function (33, 47, 56). Consistent with that idea, antigen presentation between naïve CD4$^+$ T cells and DCs upregulates T cells expression of ChAT mRNA, ChAT activity, ACh, AChE, and M_5 mAChR, thereby enhancing cholinergic activity [see reviews in Ref. (11, 12, 33, 47)].

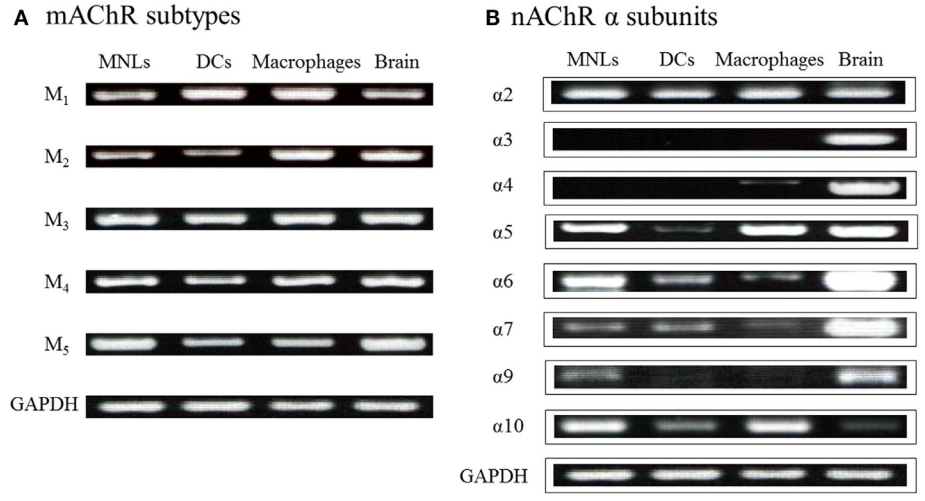

FIGURE 2 | Expression of genes for mAChRs and nAChR α subunits in immune cells from C57BL/6J mice. **(A)** mRNA expression of mAChR subtypes detected using reverse transcription-polymerase chain reaction. MNLs, mononuclear leukocytes; DCs, dendritic cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **(B)** mRNA expression of nAChR α subunits. Arranged from study by Kawashima et al. (36).

AChE and ChE

Both AChE and BuChE hydrolyze ACh into choline and acetate to terminate its activity at synapses. In the brain, AChE is mostly found within synaptic clefts between neurons (57, 58), while BuChE is mainly located outside the synaptic cleft and in glial cells (59, 60). Both AChE and BuChE are present at the mouse neuromuscular junction, but exhibit different localization patterns. AChE activity is present in both the primary cleft and in the secondary folds, while BuChE activity appears to be concentrated in structures resembling subsynaptic folds (61). AChE is also found within erythrocytes, but its physiological function there is unknown. BuChE is found primarily in plasma, liver, and the neuromuscular junction [see a review in Ref. (12)].

The decay time constants of focally recorded miniature endplate currents caused by ACh at the neuromuscular junction are 1.04 and 5.4 ms in wild-type (WT) and AChE-KO mice, respectively (62). It is important to note the differences in the inactivation rates and processes between ACh and other neurotransmitters, such as norepinephrine (NE) and epinephrine (EPI). Whereas the action of ACh is terminated within a few milliseconds through enzymatic breakdown, the actions of NE and EPI last much longer until reuptake into nerve terminals and surrounding tissues, and the diffusion decrease their concentrations within the synaptic cleft to subthreshold levels. As a consequence, to have a physiological action, non-neuronal ACh must be released into a microenvironment forming synapse-like structures, such as during antigen presentation and cell-to-cell interaction involving cell adhesion molecules.

AChE is expressed ubiquitously in mouse lymphocytes, DCs, and macrophages (36), while human blood MNLs, CEM human leukemic T cells, and Daudi B cells all express various types of AChE mRNA [see a review in Ref. (10)]. In addition, upregulation of AChE activity by PHA is detected in normal peripheral blood human lymphocytes and in leukemic T cell lines (55). These findings indicate that T cell activation *via* TCR/CD3-mediated pathways enhances expression of cholinergic elements, including ChAT and AChE, within T cells. However, the physiological function of AChE in immune cells has yet to be investigated.

Rivastigmine, which inhibits both AChE and ChE activities, relieves the clinical symptoms and spatial memory deficits in mice with autoimmune encephalomyelitis (EAE) (63). Rivastigmine also decreases the reactivity of encephalitogenic T cells and the production of TNF-α, interferon (IFN)-γ, and IL-17 cytokines in the EAE mouse. All of these effects are abolished by α-BTX, an α7 nAChR antagonist, which suggests that the effects are induced by ACh acting on α7 nAChRs after its levels were increased due to AChE and BuChE inhibition (63).

It has been suggested that functional defects in nAChRs on immune cells contribute to the etiology of inflammatory bowel diseases (IBD), as smoking modifies the development and progression of IBD (64, 65) and immunosuppressants are sometimes effective in patients with IBD (66–68). In addition, AChE-targeting microRNA-132 (miR-132) exhibited some potential to attenuate inflammation by reducing AChE levels in immune cells (69).

AChRs

Early functional and binding studies revealed the presence of both mAChRs and nAChRs on immune cells [see reviews in Ref. (9, 11, 12, 16)].

mAChR Subtypes

The diversity of mAChR functions prompted investigation of their molecular basis using cloning techniques and led to the identification of five distinct mAChR subtypes (M_1–M_5) (70–72). These five mAChRs have been divided into two groups based on their functional coupling. The M_1, M_3, and M_5 subtypes are coupled to pertussis toxin-insensitive $G_{q/11}$ proteins, which mediate activation of PLC activity. Upon activation of these mAChR subtypes, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate, leading to the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. These products then act as second messengers by, respectively, mobilizing Ca^{2+} from intracellular stores and activating PKC (73). The M_2 and M_4 subtypes are coupled to pertussis toxin-sensitive $G_{i/o}$ protein, which mediates inhibition of adenylate cyclase, and thus a decrease in cAMP formation, upon activation (74, 75).

mAChR Expression in Immune Cells

All five mAChRs subtypes have been detected in lymphocytes, macrophages, and DCs from humans, mice, and rats (**Figure 2A**) [see reviews in Ref. (16, 36, 76)]. As mentioned, PHA and SAC selectively upregulate expression of M_5 mAChR mRNA in CEM human leukemic T cells and Daudi B cells, respectively (33, 47). Expression of other mAChR subtypes is not affected in either cell line. Stimulation with PMA plus ionomycin upregulates expression of both M_3 and M_5 mAChR mRNA in these cell lines (47). These findings show that immunological stimulation leads to M_5 mAChR gene expression in lymphocytes and suggest a role for M_5 mAChRs in the regulation of immune function.

Qian et al. (77) showed that activation of murine splenic T cells for differentiation using monoclonal antibodies against CD3 and CD28 modifies the expression patterns and intensities of mAChRs. These findings suggest the possibility that the expression patterns and intensities of mAChR expression in immune cells may vary among individuals, depending on their immunological status.

nAChRs

Nicotinic AChRs are located on the plasma membranes of skeletal muscle cells, neurons, and non-neuronal cells. Activation of nAChRs elicits membrane depolarization and excitation due to a rapid increase in membrane permeability to Na^+, K^+, and Ca^{2+}. The molecular cloning of nAChR subunits (78) prompted a series of proteomic and genomic investigations of the various nAChR subunit proteins. At present, 10 α (α1–10), 4 β (β1–4), γ, δ, and ε nAChR subunits have been detected through molecular cloning. The α subunits can be subdivided according to their sensitivity to α-BTX: α1, α7, and α9 are α-BTX-sensitive, while α2–α6 are α-BTX-insensitive. nAChRs exist as pentamers composed of 1–5 different subunits. At least two copies of the α subunit are always present among these subunits, and multiple ACh binding sites are formed at the interface of each α subunit and a neighboring subunit.

nAChRs are classified into muscle and neuron types based on their expression sites. Muscle type nAChRs are expressed mainly in skeletal muscle and contain four different subunits within the pentameric complex: $(\alpha1)_2/\beta1/\delta/\gamma$ in embryonic and denervated muscle, and $(\alpha1)_2/\beta1/\delta/\varepsilon$ in innervated adult muscle. By contrast, neuron type nAChRs expressed in the nervous system and non-neuronal cells, including immune cells, are composed of only α (α2–α7, α9, and α10) and β (β2–β4) subunits. The α8 subunit is found only in the visual areas of the avian brain (79). nAChRs consisting of α3β2 and α3β4 are abundantly expressed in peripheral ganglia, while α4β2 subunits are expressed in the brain (80). Within the mammalian brain, about 80% of nAChRs are composed of α4β2 subunits, and 10–15% are composed of α7 (81). The α7 nAChR gene *(CHRNA7)* is expressed widely in the central and peripheral nervous system, and in non-neuronal cells, and the homomeric α7 nAChR exhibits uniquely high Ca^{2+} permeability upon activation (82, 83). The α9 and α10 subunits were first identified in the mechanosensory hair cells of the rat auditory system (84, 85). The α9 subunit was initially postulated to form a homomeric nAChR. However, subsequent studies identified expression of heteromeric α9α10 nAChR subtypes within the hair cells. Unlike other nAChRs, which mediate excitatory neurotransmission, α9α10 nAChR activation elicits hair cell hyperpolarization evoked by Ca^{2+} entry through the receptor, leading to activation of a small-conductance SK2 Ca^{2+}-dependent potassium channel (85).

Specific Features Related to α7 nAChRs

Human-Specific dupα7 nAChRs. The α7 subunit gene *CHRNA7* is composed of 10 exons, encoding 146 amino acids (exons 1–4) comprising an N-terminal extracellular domain and 384 amino acids (exons 5–10) comprising three transmembrane (M1–M3) domains, a large intracellular loop (M3–M4 loop), a fourth transmembrane (M4) domain, and a short C-terminal extracellular region (86). Gault et al. (87) found *CHRNA7* exons 5–10 duplicated as the *CHRFAM7A* gene encoding dupα7 nAChR in the human genome and its expression in the brain. Although the α7 nAChR and dupα7 nAChR subunits share the same 384 amino acids comprising the four transmembrane domains of the ligand-gated ion channel transmembrane region and a short C-terminal extracellular region, the dupα7 subunit has a shorter N-terminal extracellular domain than the primal α7 nAChR subunit. Because the extracellular N-terminal region of the α7 nAChR contains multiple loops of agonist binding site (88), its structure suggests that the dupα7 nAChR subunit may lack the recognition sites for ACh and α-BTX. Experiments performed with *Xenopus* oocytes co-injected with various ratios of α7/dupα7 mRNA revealed a graded reduction in functional receptor generation proportional to the α7/dupα7 ratio, as measured based on nicotine-elicited α7 currents (89). These results were confirmed by measurements of α-BTX binding, which suggests that as the proportion of the dupα7 subunit increases, there is a reduction in the number of functional α7 receptors that reach the surface of the oocyte (89). These results are consistent with the findings of Araud et al. (86), who reported that *CHRFAM7A* functions as a dominant negative regulator of α7 nAChR.

Human-specific *CHRFAM7A* transcripts were first discovered in the brain (87), but were later detected in human peripheral blood leukocytes, including MNLs (90), macrophages (90, 91), and monocytic cell lines (90, 92). The role of the dupα7 subunit in the regulation of immune function remains to be determined.

Heteromeric α7β2 nAChRs. Although the α7 subunit has long been postulated to form a homomeric α7 nAChR, recent studies suggest the possibility that α7 and β2 subunits form heteromeric α7β2 nAChR subtypes in the brain (93–95). Differences in the pharmacological and functional properties between naturally occurring α7-containing nAChRs in the brain and those of recombinant homomeric α7 nAChRs prompted studies investigating whether the nAChR α7 and β2 subunits can co-assemble to form a functional heteromeric nAChR channel in *Xenopus* oocytes and cell lines (93–95). Detection of co-expressed rat α7 and β2 subunits in co-transfected TSA201 embryonal kidney cells and SH-EP1 human epithelial cells, and of α7 and β2 subunit mRNAs in the rat cholinergic neurons, confirmed their co-assembly in mammalian cells (93, 96, 97). Furthermore, the co-expression significantly slowed the rate of channel desensitization, compared to homomeric α7 channels, and altered the pharmacological properties of the channels. It thus appears that rat nAChR α7 and β2 subunits have the ability to co-assemble and form functional heteromeric nAChRs (94, 95, 98).

Expression of α7β2 nAChRs was confirmed in the human brain through purification of α7 subunit-containing proteins using α-BTX beads followed by western blotting using an anti-β2 subunit antibody (99). Thomsen et al. (99) also found that upon agonist stimulation, the currents carried by heteromeric α7β2 nAChRs show markedly slower rising and decay phases than homomeric α7 nAChRs in HEK293 cells. At present, no information is available on the expression of α7β2 nAChRs in immune cells. Because T and B cells, DCs, and macrophages all express α7 nAChRs (36, 98, 100), it is possible that α7β2 nAChRs are also expressed in immune cells and are involved in regulating immune function.

Ionotropic and Metabotropic Natures of α7 nAChRs. As described, activation of α7 nAChRs using ACh or nicotine elicits a transient increase of $[Ca^{2+}]_i$ in neurons and immune cells, though in certain types of immune cells the receptor channel rapidly desensitizes, and no transient increase of $[Ca^{2+}]_i$ is observed (101). Nevertheless, activation of α7 nAChRs can set into motion more prolonged signaling events operating downstream and leading to modulation of immune cell function. Prompted by these observations, the dual ionotropic/metabotropic nature of α7 receptors has been extensively explored to explain the role of α7 receptors (102).

(1) *Ionotropic pathway*: the Ca^{2+} influx through α7 nAChRs induced by stimulation with ACh and agonists activates a phosphorylation cascade *via* PKC. This in turn activates the PI3K/Akt pathway, which promotes nuclear factor erythroid 2-related factor 2 (Nrf2) translocation to the nucleus and overexpression of heme oxygenase 1, resulting in potent anti-inflammatory effects (67, 102–104).

(2) *Metabolic pathway*: evidence now suggests that α7 nAChRs may interact with G-proteins independently of G-protein coupled receptors (102, 105–108). By showing the binding of G-proteins to G-protein-binding sites located in the M3–M4 loop of α7

nAChRs, King et al. (108) demonstrated that direct coupling of α7 nAChRs to G-proteins makes it possible to elicit downstream Ca^{2+} signaling responses that can persist beyond the expected time course of channel activation. It has been suggested that α7 nAChRs coupled with G_s, $G_{q/11}$, and $G_{i/o}$ proteins locating in the hippocampus and prefrontal cortex of C57BL/6J mice contribute to the regulation of neurite growth (101, 106). In addition, Razani-Boroujerdi et al. (101) found that a functional TCR/CD3 complex and leukocyte-specific tyrosine kinase are required for the nicotine-induced rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked in the absence of extracellular Ca^{2+} *via* α7 nAChRs in T cells. This suggests an alternative metabotropic pathway leading to induction of Ca^{2+} signaling in human T cells. Agonist binding to α7 nAChRs also may activate Janus kinase 2/signal transducer and activator of transcription 3 signaling cascades independently of Ca^{2+} influx, leading to suppression of nuclear transcription factor kappaB-regulated transcriptional activity in macrophages (67, 102–104).

Specific Features Related to α9 and α10 nAChRs

Hecker et al. (109) reported that ACh, choline, phosphocholine, phosphocholine-modified LPS from *Haemophilus influenzae*, and phosphocholine-modified protein all inhibit ATP-mediated IL-1β release independently of the extracellular Ca^{2+} influx through the channel in human monocytic U937 cells expressing the α7, α9, and α10 nACR subunits, and in rat monocytes *via* nAChR-mediated pathways. Furthermore, using U937 cells, Richter et al. (110) found that choline and phosphocholine inhibit ATP-mediated P2X7 receptor activation and IL-1β release. While choline elicits ionotropic current responses at homomeric α9 nACRs, phosphocholine does not trigger ionotropic responses at either homomeric α9 or heteromeric α9/α10 nAChRs. These findings provide evidence that phosphocholine and their derivatives are able to function as metabotropic agonists for heteromeric α9/α10 nAChRs.

nAChR Expression in Immune Cells

Figure 2B shows the mRNA expression for nAChR α subunits in MNLs, DCs, and macrophages from C57BL/6J mice (36). Expression of the α2, α5, α6, α7, α9, and α10 subunits is frequently detected in immune cells, including T cells (16, 76, 111, 112). However, the specific patterns of nAChR subunit expression in particular immune cells are not yet settled. As mentioned, it appears that the patterns and the intensities of nAChR subunit expression vary depending on the immunological status of the cell in question (77). It is noteworthy, however, that data from immunohistochemistry and western blots must be interpreted cautiously due to the lack of the specificity of the commercially available antibodies (113, 114).

SLURP-1 and -2: Endogenous Positive Allosteric Ligands for α7 and α3 nAChRs

SLURP-1 and -2 are endogenous peptides that act as positive allosteric ligands on α7 and α3 nAChRs, respectively (115–119). The discovery of mutations in the gene encoding SLURP-1 in Mal de Meleda (MdM) patients with a characteristic transgressive palmoplantar keratoderma (120) is drawing major research attention to the capacity of SLURP-1 to serve as an epithelial growth modulator (112, 118–122). In addition, recent studies suggest the possibility that SLURP-1 and -2 are also involved in regulating immune cell function (36, 122–126).

Gene Expression for SLURP-1 and -2

SLURP-1 and -2 mRNAs are detected in nearly every organ in the C57BL/6J mouse (123). Gene expression for SLURP-1 and -2 was also detected in MNLs, DCs, and macrophages, but neither the T cell activator Con A nor the DC and macrophage activator LPS modified the levels of their expression (36, 123). This suggests that SLURP-1 and -2 are constitutively expressed in these cells, and their expression is independent of immunological stimulation.

Immunoreactive SLURP-1 Expression in Immune Cells

Intense SLURP-1 immunoreactivity (SLURP-1^+) was detected in DC-like cells residing mainly in the interfollicular zone surrounding the germinal center of human tonsils, and in a few cells scattered within the germinal center (**Figure 3**) (126). Some SLURP-1^+ cells in the tonsil interfollicular zone also showed immunopositivity for CD205 (**Figure 3A**), a marker of mature DCs that mediates efficient antigen presentation (127), and these SLURP-1^+ $CD205^+$ DCs were surrounded by $CD4^+$ T cells. These observations support the notion that ACh synthesized and released from T cells and activated macrophages during antigen presentation acts on α7 nAChRs expressed in immune cells, and that SLURP-1 potentiates the ACh activity at α7 nAChRs in both T cells and DCs (16, 126).

ROLE OF THE CHOLINERGIC SYSTEM IN THE REGULATION OF IMMUNE FUNCTION

Roles of mAChRs in the Regulation of Immune Cell Function

Agonist-evoked activation of mAChRs in immune cells evokes a variety of functional and biochemical effects, including enhanced cytotoxicity, increased cGMP and IP_3 formation, and activation of cell proliferation [see reviews in Ref. (9, 12)]. Stimulation of mAChRs in CEM human leukemic T cells and Daudi human leukemic B cells using ACh, bethanechol, carbachol, or Oxo-M induced a transient rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by extracellular Ca^{2+}-dependent $[Ca^{2+}]_i$ oscillations that persisted for about 10 min (128). In both cell lines, Oxo-M upregulated c-fos mRNA expression in an extracellular Ca^{2+}-dependent manner. All these effects induced by mAChR agonists were abolished by atropine, a non-specific mAChR antagonist. This suggests that activation of mAChRs in immune cells triggers nuclear signaling, leading to modification of immune cell function. More recently, Mashimo et al. (54) showed that M_3 and M_5 mAChRs play a major role in Oxo-M-induced initial transient increases in $[Ca^{2+}]_i$ and the following repetitive $[Ca^{2+}]_i$ oscillations in CEM human leukemic T cells. The $[Ca^{2+}]_i$ oscillations were blocked by removal of extracellular Ca^{2+} or YM-58483, a CRAC channel blocker, without affecting

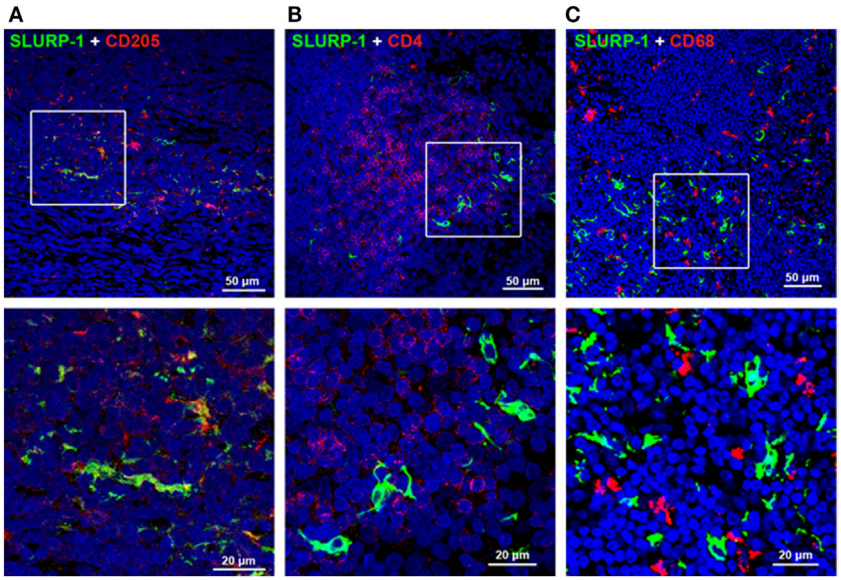

FIGURE 3 | Immunohistochemical staining of SLURP-1, CD205, CD4, and CD68 in human tonsils. **(A)** Merged image showing SLURP-1 (green) and CD205 (red) immunoreactivities in the interfollicular zone. Note that some $CD205^+$ cells were also $SLURP\text{-}1^+$. An enlarged image of the boxed area is shown in the lower panel. **(B)** Merged image showing SLURP-1 (green) and CD4 (red) immunoreactivities in the interfollicular zone. Note that the $SLURP\text{-}1^+$ cells are surrounded by $CD4^+$ T cells. An enlarged image of the boxed area is shown in the lower panel. **(C)** Merged image showing SLURP-1 (green) and CD68 (red) immunoreactivities in the interfollicular zone. Note that the $SLURP\text{-}1^+$ cells are located in close proximity to $CD68^+$ macrophages. An enlarged image of the boxed area is shown in the lower panel. Arranged from data by Fujii et al. (126).

the Oxo-M-induced initial $[Ca^{2+}]_i$ transient. Furthermore, CRAC channel blockade abolished Oxo-M-induced c-fos and IL-2 expression. These results suggest that activation of M_3 or M_5 mAChRs evokes IP_3-mediated Ca^{2+} release from intracellular stores, leading to extracellular Ca^{2+} influx through CRAC channels, which generates repetitive $[Ca^{2+}]_i$ oscillations and, in turn, enhances c-fos and IL-2 gene expression in T cells.

M_1 and M_5 mAChRs in Cytolytic Activity

Upregulation of M_5 mAChR mRNA expression in human T and B cells during immunological stimulation strongly suggests that mAChRs, including M_5, are involved in regulating immune function (33, 47). Thus far, however, there have been few investigations of the roles played by mAChRs in regulation of immune function. The involvement of M_1 mAChRs in the differentiation of $CD8^+$ T cells into cytolytic T cells was first postulated by Zimring et al. (129), but it was later found that neither M_1 nor M_5 mAChRs are required for expansion of antigen-specific $CD8^+$ T cells, in response to viral infection in C57BL/6 mice (130). On the basis of these observations, the authors concluded that the extent to which one can draw a generalized conclusion that M_1 and M_5 mAChRs are not involved in antiviral immunity mediated by $CD8^+$ T cells, depends upon issues of antigen strength, genetic background, and receptor redundancy (130).

M_1 and M_5 mAChRs in Antibody and Cytokine Production

Fujii et al. (56) investigated the roles of M_1 and M_5 mAChRs in the regulation of immune function by immunizing combined M_1 and M_5 mAChR gene-knockout (M_1/M_5-KO) and WT mice with ovalbumin (OVA). One week after the immunization, serum concentrations of anti-OVA-specific IgG_1 in the M_1/M_5-KO mice were significantly lower than in WT mice, though the serum concentrations of anti-OVA-specific IgM did not differ between the two genotypes. Spleen cells from M_1/M_5-KO mice activated with OVA secreted significantly lower amounts of TNF-α, IFN-γ, and IL-6 than those from WT mice. These observations suggest that M_1 and/or M_5 mAChRs contribute to the regulation of pro-inflammatory cytokine production related to adaptive immunity.

Roles of nAChRs in the Regulation of Immune Function

Among the various nAChRs, α7 nAChRs are drawing attention because their stimulation attenuates the synthesis and release of the pro-inflammatory cytokine TNF-α in LPS-activated macrophages, leading to modulation of inflammatory and immune responses (15, 131, 132).

Upregulation of Antibody and Pro-Inflammatory Cytokine Production in *CHRNA7* Knockout (α7-KO) Mice

Fujii et al. (133) observed a significantly higher serum anti-OVA-specific IgG_1 levels in α7-KO than WT C57BL/6J mice 2 weeks after immunization with OVA. Moreover, antigen-stimulated spleen cells from α7-KO mice produced significantly greater amounts of TNF-α, IL-6, and IFN-γ responsible for antibody class switch induction to IgG_1 than those from WT mice.

These observations suggest the involvement of α7 nAChRs in regulating pro-inflammatory cytokine production, leading to modification of antibody production. In line with these findings, a recent study showed that nicotine attenuates production of TNF-α, IL-1β, and IL-12 in murine bone marrow-derived monocytes *via* α7 and α9 nAChR-mediated pathways (134). In addition, activation of B cells with anti-CD40 antibody elicited a greater proliferative response in α7-KO mice than in the WT, and the suppression of α7 nAChRs with methyllycaconitine (MLA) evoked a greater proliferative response in B cells stimulated either with anti-CD40 antibody or antibody against B cell receptor (135–138), suggesting a role for α7 nAChRs in downregulation of B cell proliferation. Taken together, the above findings suggest the possibility that a higher serum antigen-specific IgG_1 concentration observed in α7-KO mice (133) can be ascribed to the upregulation of pro-inflammatory cytokine production and the increased number of B cells in α7-KO mice (135–138).

Roles of nAChRs in IL-2 Production and Proliferation of T Cells

MOLT-3 human leukemic T cells and cultured spleen cells from C57BL/6J mice constitutively produce amounts of ACh sufficient to elicit autocrine changes in $[Ca^{2+}]_i$ and upregulation of IL-2 mRNA and protein expressions (139). Mecamylamine, a nAChR inhibitor, suppressed the $[Ca^{2+}]_i$ transients, IL-2 release, and cell proliferation. These findings indicate that T cells utilize ACh as a tool to interact with one another and that autocrine ACh-activated nAChRs are involved in regulating immune cell functions such as cytokine synthesis and cell proliferation.

Methyllycaconitine, a specific α7 nAChR antagonist, did not suppress the above described autocrine ACh-induced changes in $[Ca^{2+}]_i$, suggesting the involvement of nAChRs other than α7 nAChRs in the $[Ca^{2+}]_i$ transients in freshly isolated spleen cells and resting MOLT-3 cells leading to upregulation of IL-2 production (139). In fact, Qian et al. (77) detected the expression of α7 nAChR mRNA in $CD4^+$ and $CD8^+$ T cells only after the activation *via* TCR/CD3 cross-linking, but not in freshly isolated $CD4^+$ and $CD8^+$ T cells. Taken together, these findings suggest the possibility that the pattern of nAChR subtype expression is variable depending on the immunological status. Furthermore, nicotine modified IFN-γ and IL-17 production in T cells activated with TCR/CD3 cross-linking (77, 140). These findings suggest that nAChRs including α7 nAChR contribute to immunomodulation through modification of T cell differentiation by altering cytokine production.

Roles for SLURP-1 in the Regulation of Immune Cell Function

T Cell Activation

T cell activation with anti-CD3/anti-CD28 mAbs of peripheral blood MNLs isolated from MdM patients with SLURP-1 mutation showed a defect in their proliferative response (125). Moreover, addition of WT recombinant SLURP-1 (rSLURP-1) to cultures of T cells from MdM patients restored the normal T cell activation response, showing that SLURP-1 plays a key role during normal activation of T cells induced by immunological stimulation enhancing the actions of ACh *via* α7 nAChRs (125).

ACh Synthesis in T Cells

Recombinant SLURP-1 increases ChAT gene expression and the ACh content in MOLT-3 human leukemic T cells and human peripheral blood MNLs, and these effects are abolished by the α7 nAChR antagonist MLA. This suggests that, working as a positive allosteric ligand, SLURP-1 activates cholinergic transmission by potentiating ACh synthesis and its action on α7 nAChRs, thereby facilitating functional development of T cells (126). rSLURP-1 induces a slight but significant attenuation of cell growth in peripheral blood MNLs and MOLT-3 cells and that is abolished by MLA. These findings support the notion that SLURP-1 acts as a key modulator of T cell activity.

Effects of SLURP-1 and -2 on Immune Cells

Chernyavsky et al. (122) found that rSLURP-1 decreases production of TNF-α in CEM human leukemic T cells, downregulates IL-1β and IL-6 secretion in U937 macrophages, and moderately upregulates IL-10 production in these immune cells. rSLURP-2 downregulates TNF-α and IFN-γ-receptors in CEM cells and reduces IL-6 production in U937 macrophages. These results demonstrate that SLURP-1 and -2 exert anti-inflammatory effects on T cells and macrophages.

Roles for α7 nAChRs in T Cell Differentiation

As described, α7 nAChRs on immune cells are involved in regulating the synthesis of various cytokines (16, 131, 133). On the basis of those findings, Kawashima et al. (141) used α7-KO and WT mice to investigate the involvement of α7 nAChRs on naïve $CD4^+$ T cells in the regulation of their differentiation to $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Tregs). Non-specific activation of naïve $CD4^+$ T cell differentiation in culture using anti-TCR and anti-CD28 mAbs in the presence GTS-21, a partial α7 nAChR agonist, upregulated the generation of Tregs from WT spleen cells, but not from α7-KO cells (**Figure 4**). On the other hand, assays of IFN-γ in the culture media suggested that Th1 differentiation was not affected by α7 nAChR activation. This suggests that when naïve $CD4^+$ T cells are directly stimulated *via* their TCR/CD3 complex, activation of α7 nAChRs on the T cells leads to upregulation of Treg and that α7 nAChRs are also involved in regulating adaptive immunity.

Studies examining the effects of adoptive transfer of antigen-specific Tregs have demonstrated their contribution to the protection and recovery of an animal model of autoimmune EAE (144). However, the availability of adequate numbers of antigen-specific Tregs for adoptive transfer is difficult to achieve and is one of the major limitations of its clinical application. For treatment of autoimmune diseases, it would be useful to develop procedures in which immune cell cholinergic activity was used to facilitate Treg differentiation from naïve $CD4^+$ T cells. Conversely, suppression of Treg differentiation through manipulation of cholinergic

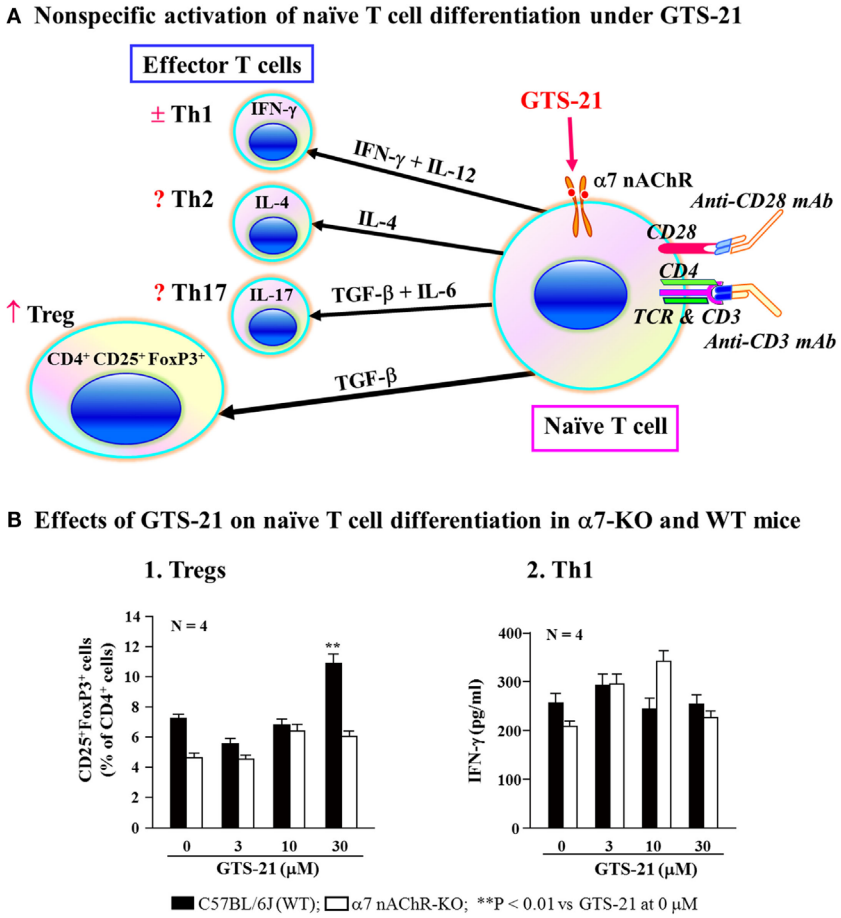

FIGURE 4 | Role for α7 nAChRs during differentiation of naïve T cells. **(A)** Schematic drawing of non-specific activation of naïve T cell differentiation *via* T cell receptor and CD28 in the presence of GST-21, a partial agonist for α7 nAChRs. Differentiation of naïve CD4+ T cells into effector T cells (Th1, Th2, and Th17) and regulatory T cells (Tregs) is dictated by the presence of cytokines shown on the respective arrows (142, 143). GTS-21 may facilitate the expression of transcription factor FoxP3 or TGF-β leading to Treg expansion. IFN-γ, interferon-γ; IL-4, interleukin-4; IL-6, interleukin-6; IL-12, interleukin-12; IL-17, interleukin-17; TGF-β, transforming growth factor-β. **(B)** Effects of GST-21 on naïve T cell differentiation in α7 nAChR-deficient (α7-KO) and wild-type (WT) mice. Note that GTS-21 upregulated differentiation into Tregs in the WT, but not α7-KO mice. Th1 differentiation was not affected by GTS-21 in the both genotypes. Arranged from study by Kawashima et al. (141).

activity in immune cells could potentially be of great help in cancers therapy.

INFLAMMATORY REFLEX IN THE CONTEXT OF THE IMMUNE CELL CHOLINERGIC SYSTEM

Inflammatory Reflex

The inflammatory reflex involves the nervous systems and humoral factors that operate to protect the body from infection and inflammation. Pro-inflammatory cytokines generated by immune cells in response to invading pathogens trigger a series of reflex responses. The inflammatory reflex loop consists of two major components: (1) a sensory afferent signaling pathway and (2) a motor efferent signaling pathway. The afferent signaling pathway conveys peripheral signals to the brain *via* the afferent sensory vagus nerve and humoral routes. After the signals are processed in the brainstem, the central nervous system transmits signals to the periphery *via* (1) humoral pathways that involve activation of the hypothalamic–pituitary–adrenal axis, leading to the release of glucocorticoids with anti-inflammatory actions (13), (2) neural pathways involving activation of the sympathetic nervous system (13, 14), and (3) the efferent vagus nerve (16, 145–147). Here, we will mainly touch on the efferent signaling pathways of the inflammatory reflex in the context of the immune cell cholinergic system.

Background of an Anti-Inflammatory Reflex

Intraperitoneal injection of IL-1β, a pro-inflammatory cytokine, induces fever that is dependent on intact afferent vagus neurons (148). In a rat model of LPS-induced septic shock, electrical stimulation of the efferent vagus nerve protected animals from endotoxemia and attenuated the increase in serum and liver

TNF-α levels (149). In addition, ACh and nicotine abolished the LPS-induced release of TNF-α from cultured human macrophages. Electrical stimulation of the vagus nerve also inhibited LPS-induced TNF-α synthesis in WT mice, but failed to inhibit TNF-α synthesis in α7-KO mice, suggesting that α7 nAChRs are essential for inhibiting cytokine synthesis *via* a cholinergic anti-inflammatory pathway (131, 150–152).

Following the observations that splenectomy and selective abdominal vagotomy abolished the anti-inflammatory effects of both vagus nerve stimulation and nicotine, the spleen was identified as an essential target of the cholinergic anti-inflammatory pathway involved not only in inflammatory signal generation in the afferent vagus nerve but also vagally mediated inhibition of cytokine production in macrophages (153–155).

Although the attenuation of LPS-induced septic shock by efferent vagal stimulation suggests the involvement of the vagus nerve in the anti-inflammatory reflex (150), the pathways and mechanisms that carry efferent inhibitory signals suppressing the synthesis and release of pro-inflammatory cytokines in the spleen are not yet settled. There is no neuroanatomical evidence for a direct parasympathetic or vagal nerve supply to any immune organ (156). The currently available evidence indicates that all primary and secondary immune organs receive innervation only by sympathetic postganglionic neurons [see a review by Nance and Sanders (14)]. Prevertebral sympathetic ganglia associated with the celiac-mesenteric plexus provide major sympathetic input to the spleen, and the splenic nerve is the final common pathway for neural input to the spleen (14, 157).

Currently Proposed Efferent Signaling Pathways in the Anti-Inflammatory Vagal Reflex

Anti-Inflammatory Vagal Reflex Mediated by Noradrenergic Splenic Nerve and ChAT$^+$ T Cells in the Spleen

Because the efferent vagus nerve that should carry the efferent signals terminates in the celiac ganglion, Rosas-Ballina et al. (15) postulated that the efferent signals carried by the efferent vagus nerve are transmitted to the postganglionic sympathetic splenic nerve innervating the spleen in the celiac ganglion. This would activate the splenic nerve to release NE within the spleen, which would promote ACh release from a subset of CD4$^+$ T cells that relay the neural signal to other immune cells—e.g., through activation of α7 nAChRs on macrophages, resulting in inhibition of the synthesis and release of pro-inflammatory cytokines, including TNF-α. Consistent with those ideas, they found that electrical vagus stimulation elevated ACh concentrations in the spleen, and attenuated serum TNF-α levels during endotoxemia. Their observations further confirmed that T cells mediate the inflammatory reflex. They found that vagus nerve stimulation did not diminish serum TNF-α concentrations during endotoxemia in nude mice lacking functional T cells, but that the transfusion of a subset of ChAT$^+$ T cells from normal mice to the nude mice restored the ability of vagus nerve stimulation to suppress serum TNF-α concentrations. They, therefore, proposed that efferent vagus nerve signals are transferred to ChAT$^+$ T cells *via* NE released from the splenic nerve in the spleen, leading to elevation of ACh synthesis through activation of β-adrenoceptors on the ChAT$^+$ T cells. The elevated ACh in turn acted on α7 nAChRs to suppress synthesis and release of TNF-α in macrophages (15).

Anti-Inflammatory Vagal Reflex Mediated by Recruited ChAT$^+$ T Cells and NE Released from Sympathetic Nerve Terminals in the Spleen

Based on the observation that electrical stimulation of the vagus nerve did not elicit action potentials in the splenic nerve (158), Martelli et al. (159) proposed that efferent vagal stimulation and/or antidromic vagal afferent activation elicits lymphocyte mobilization to the spleen from a deposit of lymphocytes (vagal target). Then within the spleen, ACh-synthesizing CD4$^+$ T cells among the mobilized lymphocytes release ACh to activate α7 nAChRs on the sympathetic nerve terminals leading to NE release. NE released from sympathetic nerve terminals acts on β_2-adrenoceptors on macrophages to suppress TNF-α synthesis and release [see reviews in Ref. (13, 14)]. However, considering the extreme enzymatic fragility of ACh, it is essential to confirm the formation of synapse-like structures between ACh-synthesizing CD4$^+$ T cells and sympathetic nerve terminals in the spleen, and between vagus nerve terminals and the vagus target. It is also necessary to demonstrate α7 nAChR expression in the sympathetic nerve terminals in the spleen and their functional release of NE.

A Possible Involvement of the Cholinergic System in Immune Cells and SLURP-1 in Inflammatory Reflex

SLURP-1 acts as a positive allosteric ligand for α7 nAChRs (117, 118) and increases the potency and efficacy of ACh at α7 nAChRs more than 2.5 times (117). SLURP-1 co-localizes with subsets of sensory neurons containing substance P (SP) and calcitonin gene-related peptide (CGRP) (160), which have been detected among afferent vagus nerve fibers (161, 162). In the white pulp of the spleen, SP$^+$ nerve fibers have been identified within the marginal zone and the outer regions of the periarteriolar lymphoid sheaths, which are filled with T cells (163). In mice, CGRP$^+$ nerve fibers have been detected in the spleen and lymph nodes (164, 165). Furthermore, the significant increase in splenic CGRP levels seen during the initial swelling phase of antigen-induced arthritis in mice suggests inflammation-induced release of CGRP from the sensory neurons (166). These findings provide evidence for splenic innervation by sensory neurons containing both SP and CGRP, and suggest the possibility of co-localization of SLURP-1 in the SP and CGRP nerve fibers innervating the spleen.

A subset of CD205$^+$ DCs located within the marginal zone of human tonsils contain immunoreactive SLURP-1 and are surrounded by CD4$^+$ T cells and CD68$^+$ macrophages (126) (**Figure 3C**). Detection of SLURP-1 in human plasma, urine, sweat, saliva, and tears is suggestive of the protein's stability and mobility (167). Moreover, SLURP-1 potentiates ACh synthesis in T cells (126). It is therefore reasonable to suggest that by potentiating the action of ACh at α7 nAChRs, SLURP-1 released

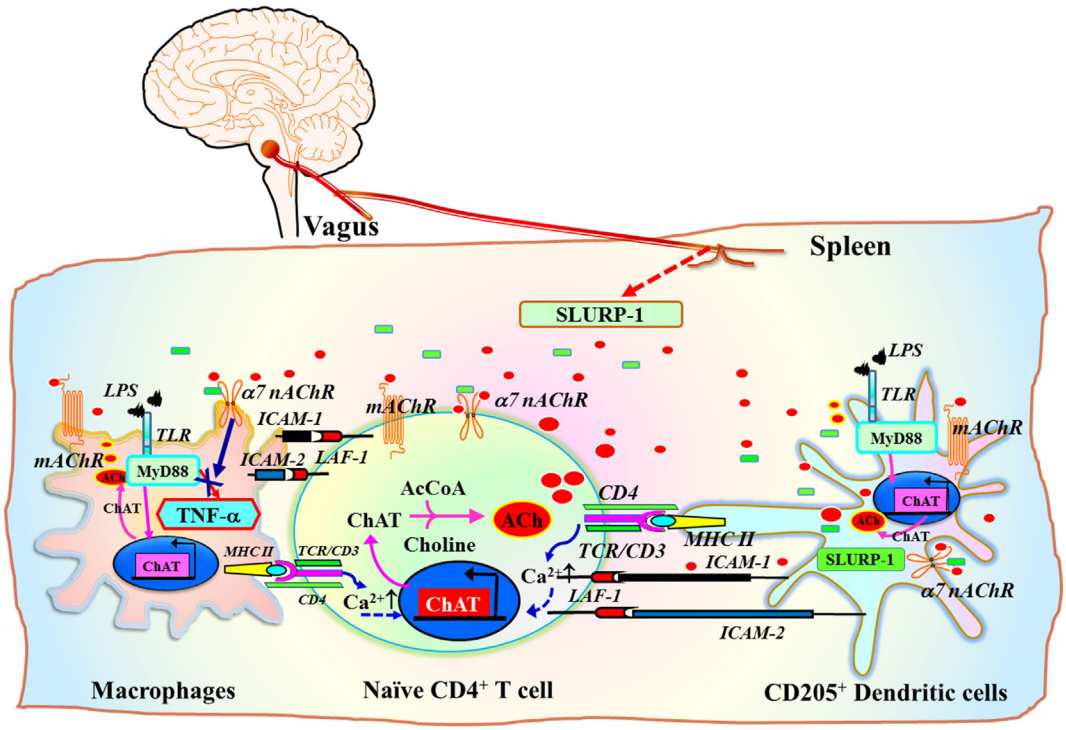

FIGURE 5 | Schematic drawing of the efferent signaling pathway of the inflammatory reflex in the context of the cholinergic system in immune cells. ChAT$^+$ T cell interaction with antigen peptides loaded on MHC class II on dendritic cells (DCs) or macrophages *via* the TCR/CD3 complex, CD80/CD86 (B7) co-stimulatory molecules with CD28, and ICAM-1/ICAM-2 with LFA-1, increases the synthesis and release of ACh from T cells [see a review by Fujii et al. (16)]. Inflammatory mediators induce the release of the positive allosteric α7 nAChR ligand SLURP-1 from SP/CGRP-containing sensory fibers. Efferent inflammatory reflex signaling *via* the vagus nerve may also induce released SLURP-1 from SP$^+$/CGRP$^+$ fibers in the spleen. This released SLURP-1, as well as SLURP-1 released from CD205$^+$ mature DCs, potentiates the action of ACh from ChAT$^+$ CD4$^+$ T cells at α7 nAChRs on macrophages, thereby suppressing synthesis and release of tumor necrosis factor (TNF)-α. The model proposed here is from reviews by Kawashima et al. (111) and Fujii et al. (16). Green rectangles depict SLURP-1. Red ellipses depict acetylcholine. AcCoA, acetyl coenzyme A; ICAM-1, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; LFA-1, lymphocyte function-associated antigen-1; LPS, lipopolysaccharide; MHC II, major histocompatibility complex class II; SLURP-1, secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide-1; TCR, T cell receptor; TLR, toll-like receptor.

from CD205$^+$ DCs during antigen presentation and from SP/CGRP-containing neurons may participate in the inflammatory reflex leading to suppression of TNF-α synthesis in splenic macrophages (**Figure 5**). Because CD4$^+$ T cells can directly interact with splenic macrophages through antigen presentation or cell-to-cell adhesion *via* LFA-1 (CD11a), ACh released from CD4$^+$ T cells should act effectively on α7 nAChRs on macrophages in the presence of SLURP-1, leading to the suppression of TNF-α synthesis and release.

CONCLUSION

Currently available evidence suggests that the immune cell cholinergic system makes a critical contribution to the regulation of immune function. For example, the cholinergic signals generated by immune cells appear to be triggers of both the initiation and termination of cytokine synthesis (e.g., IL-2 in T cells and TNF-α in macrophages). Furthermore, the evidence raises the possibility that immune function can be modulated by manipulating the cholinergic activity of immune cells. Thus, a fuller understanding of the immune cell cholinergic system could be useful for the development of drugs and therapeutic strategies for the treatment of inflammation-related diseases and cancers.

AUTHOR CONTRIBUTIONS

TF, HM, SO, and KK conceived of the review. TF and KK drafted the initial version of the manuscript and refined the contents. MM, YM, SO, and KH contributed to reference analyses, prepared tables and figures, and revised the manuscript, which was progressively edited by TF, MM, SO, and KK. TF, MM, YM, HM, SO, KH, and KK reviewed and approved the final version of this review manuscript.

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Add-on Pyridostigmine Enhances CD4+ T-Cell Recovery in HIV-1-Infected Immunological Non-Responders: A Proof-of-Concept Study

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Background: In human immunodeficiency virus (HIV)-infection, persistent T-cell activation leads to rapid turnover and increased cell death, leading to immune exhaustion and increased susceptibility to opportunistic infections. Stimulation of the vagus nerve increases acetylcholine (ACh) release and modulates inflammation in chronic inflammatory conditions, a neural mechanism known as the *cholinergic anti-inflammatory pathway* (CAP). Pyridostigmine (PDG), an ACh-esterase inhibitor, increases the half-life of endogenous ACh, therefore mimicking the CAP. We have previously observed that PDG reduces *ex vivo* activation and proliferation of T-cells obtained from people living with HIV.

Methods: We conducted a 16-week proof-of-concept open trial using PDG as add-on therapy in seven HIV-infected patients with discordant immune response receiving combined antiretroviral therapy, to determine whether PDG would promote an increase in total CD4+ T-cells. The trial was approved by the Institutional Research and Ethics Board and registered in ClinicalTrials.gov (NCT00518154).

Results: Seven patients were enrolled after signing informed consent forms. We observed that addition of PDG induced a significant increase in total CD4+ T-cells (baseline = 153.1 ± 43.1 vs. week-12 = 211.9 ± 61.1 cells/μL; $p = 0.02$). *Post hoc* analysis showed that in response to PDG, four patients (57%) significantly increased CD4+ T-cell counts (responders = 257.8 ± 26.6 vs. non-responders = 150.6 ± 18.0 cells/μL; $p = 0.002$), and the effect persisted for at least 1 year after discontinuation of PDG.

Conclusion: Our data indicate that in patients with HIV, add-on PDG results in a significant and persistent increase in circulating CD4+ T-cells.

Keywords: HIV, pyridostigmine, CD4+ T-cell, immune reconstitution, cholinergic anti-inflammatory pathway

INTRODUCTION

Effective suppression of viral replication with the use of antiretroviral therapy (cART) leads to recovery of circulating CD4+ T-cells (1, 2). Around 20% of patients starting cART with advanced disease show an insufficient increase of CD4+ T-cells, even after adequate suppression of viral replication, reflecting an incomplete immune reconstitution (3, 4). Those patients, called *immunological non-responders* (INRs), have increased long-term susceptibility to opportunistic infections, as well as accelerated disease progression and mortality (5). Various mechanisms have been proposed to underlie incomplete immune reconstitution, including older age, higher baseline HIV-RNA, low baseline CD4+ counts (≤50 cells/μL), poor adherence to therapy, and enhanced CD4+ cell activation, all leading to cell dysfunction and apoptosis (6).

The cholinergic anti-inflammatory pathway (CAP) is a neural mechanism that modulates inflammation through the release of acetylcholine (ACh), resulting in decreased synthesis of inflammatory cytokines such as TNF-α and IL-1 (7). Pyridostigmine (PDG), a peripherally acting ACh-esterase inhibitor used for the treatment of patients with myasthenia gravis, increases ACh bioavailability. We recently showed that in HIV-infected patients, PDG reduces T-cell overactivation and proliferation, reduces production of IFN-γ, and increases production of IL-10 (8).

We therefore hypothesized that in HIV-infected patients with complete HIV-RNA suppression, but incomplete CD4+ T-cell reconstitution, adding PDG to cART would promote the recovery of CD4+ T-cell counts by reducing T-cell overactivation.

METHODS

Study Design

The study was investigator-generated and planned as a prospective, interventional, open label, proof-of-concept trial, to determine whether the addition of PDG to cART improved the immune status of HIV-1-infected subjects with optimal virological control but inadequate immune reconstitution. Eligible patients received PDG bromide [Mestinon®; 3-(dimethylaminocarbonyloxy)-1-methylpyridinium bromide; CAS number: 101-26-8] 30 mg, TID, PO as add-on to their usual cART for 16 weeks. At each visit, participants were asked about compliance, tolerability, and adverse effects. Then, patients received a refill of PDG to cover the following 4-week period plus a 7-day supply. Delivery of PDG was done at the outpatient Infectious Diseases Clinic.

Ethical Considerations

The trial was approved by the Institutional Review Board and by the Ethics Committee (registered as Ref-1663 and Ref-1873) of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. The study was also registered in a public registry (ClinicalTrials.gov identifier: NCT00518154). All participants signed a written informed consent, keeping a copy themselves. Valeant Pharmaceuticals donated the PDG tablets but had no role on study design, analysis, or publication of results.

Patients

We invited adult patients (≥18 years), carriers of HIV-1 infection being followed at the HIV/AIDS Clinic of our Institute. Patients had to be taking effective cART, as evidenced by suppressed viremia under a constant therapeutic regime for at least 2 years, but with incomplete immunological response. We defined incomplete immunological response as failure to increase at least 50 CD4+ cells/μL/year during the first 2 years of virological control; failure to reach a CD4+ cell count of 200/μL; or a combination of both. A flowchart including participant selection, recruitment, and analysis is presented as **Figure 1**.

A complete blood count (CBC) as well as CD4+ and CD8+ T-cell quantification were carried at pre-established time

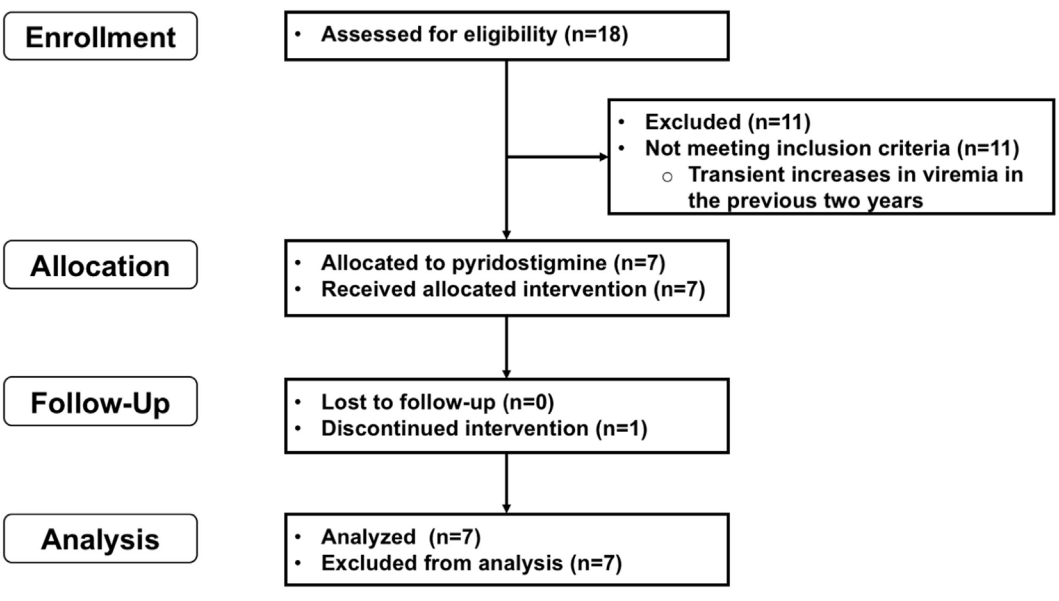

FIGURE 1 | CONSORT flowchart of patient enrollment, follow-up, and analysis.

points: 8, 12, and 16 weeks of PDG; 4 weeks after the last dose of PDG (*washout* period); and, 1 year after the last intake of PDG. Patients were blinded to their response to PDG during the study period. Exclusion criteria included the following: (a) concomitant active or neoplastic disease; (b) history of new AIDS-defining illness during cART; (c) pregnancy or breast feeding; (d) patients who had been subjects of an investigational agent, chemotherapy, or radiotherapy within the previous 28 days; (e) active tuberculosis or other opportunistic infections; (f) that participants were unable to follow or comply with the protocol interventions; and (g) that participants were receiving immunosuppressive or immunomodulatory drugs, including corticosteroids.

Measured Outcomes

The primary outcome was the number of circulating CD4$^+$ T-cells at different time points in comparison with the baseline level. Secondary readouts included (a) ratio of baseline-to-peak CD4$^+$ cell counts and (b) changes in total T-cell numbers. CD4$^+$ and CD8$^+$ T-cells were quantified by flow cytometry as described previously (8). Analysis of samples was carried at a core facility, where technicians were unaware of sample source.

Statistical Analysis

Being a proof-of-concept study, and aiming to include all eligible subjects, we had no sample size or power estimation. Exploratory data analysis was made using graphic methods. We describe baseline demographic, clinical, and laboratory characteristics of participants using means as measure of central tendency and its corresponding SDs. We did an unplanned analysis to search for factors associated with adequate immune response using the same definition of adequate response used for inclusion criteria. We carried out an intention-to-treat analysis to include the single participant who stopped experimental medication early. We present statistics of the whole group and of stratified patients according to their immune response to PDG administration. We compared the differences in CD4$^+$ T-cell concentrations at baseline, and at weeks 8, 12, and 16 using paired *t-tests* and summarize these changes using graphical methods. We compared mean measured CD4$^+$ T-cells at each time point between patients with ANOVA. Statistical analysis was done on Stata (release 12) or GraphPad Prism (version 5).

RESULTS

Patients

Eighteen patients fulfilling the inclusion criteria were screened and invited to participate. Eleven patients had had transient elevations in viremia during the 2 years before recruitment and were excluded. The remaining seven patients were enrolled after signing informed consent. All were male and infected through unprotected sex with males (MSM). All were free of co-infections, including hepatotropic viruses, tuberculosis, or opportunistic pathogens. Baseline characteristics of the patients can be found in **Table 1**. In brief, patients had been diagnosed as HIV-positive for a mean of 76.6 months (6.3 years) and, on average had delayed the start of antiretroviral therapy for almost 3 years. Except for persistently low CD4$^+$ cells (153.2 ± 43.1 cells/μL), participants had CBCs within the normal range (**Table 1**).

TABLE 1 | Baseline characteristics of participants according to response status.

	Patients		
	All (n = 7)	**Responders (n = 4)**	**Non-responders (n = 3)**
	***N* (SD)**	***N* (SD)**	***N* (SD)**
Baseline demographic data			
Age at inclusion and start of PDG (years)	48.1 (11.1)	42.6 (9.20)	55.6 (10.1)
Age at diagnosis (years)	41.7 (12.3)	37.3 (11.4)	47.6 (12.8)
Diagnosis to cART span (months)	35.0 (53.2)	26.6 (41.4)	46.2 (74.8)
HAART to PDG span (months)	41.6 (20.3)	35.9 (10.9)	49.1 (30.1)
Diagnosis to start of PDG (months)	76.6 (68.0)	62.5 (41.4)	95.4 (101.8)
Circulating CD4$^+$ T cells at diagnosis (cells/μL)	73.0 (66.2)	64.3 (40.3)	84.7 (101.8)
Circulating CD4$^+$ T cells at start of cART (cells/μL)	77.4 (43.5)	74.3 (17.6)	81.7 (71.8)
CBC at initiation of pyridostigmine			
Hemoglobin (g/dL)	15.0 (0.7)	15.5 (0.9)	14.7 (0.4)
Hematocrit (%)	42.6 (1.9)	44.4 (2)	41.7 (1.3)
WBC (×10^3/μL)	5.6 (1.8)	4.4 (0.3)	6.4 (2.0)
Granulocytes (×10^3/μL)	3.8 (1.8)	4.5 (2.2)	2.9 (0.3)
Monocytes (×10^3/μL)	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)
Lymphocytes (×10^3/μL)	1.2 (0.6)	1.4 (0.8)	1.1 (0.2)
Circulating CD4$^+$ T cells (cells/μL)	153.2 (43.1)	170.3 (43.3)	130.3 (37.0)

Data expressed as follows: mean (±SD) or median (p25–p75).
cART, combined antiretroviral therapy; PDG, pyridostigmine.

Antiretroviral Therapy and Virological Control

Four subjects were taking boosted protease inhibitor (PI)-based regimens (three lopinavir and one atazanavir), and three were receiving non-nucleoside transcriptase inhibitor (NNRTI)-based regimens (efavirenz). Five subjects were receiving their first cART regimen (three NNRTI-based and one PI-based). Throughout the study, all HIV load determinations were below detection level (Roche 1.5 ultra-sensitive).

Add-on PDG

Six patients reported full adherence to PDG. One patient (on the Responder group, as described below) took PDG for the first 12 weeks, and then withdrew consent for reasons unrelated to the study, but accepted to have CBC and CD4$^+$ determinations taken from the charts up to the predetermined 1-year posttrial time points. Adverse reactions to PDG were monitored daily during the first week, then weekly during the first month and every 4 weeks thereafter. No cardiovascular, respiratory, gastrointestinal, genitourinary, or other reactions were identified. Monitoring of the central nervous system functions did not reveal any symptom of cholinergic side effects. Compliance relied on self-report.

CD4$^+$ Response to PDG

We observed a significant increase in CD4$^+$ T-cell counts in response to the addition of PDG (baseline = 153.2 ± 43.1 vs. week-12 = 211.9 ± 61.1 cells/μL; $p = 0.02$), that persisted for as long as PDG was taken. After discontinuing PDG, circulating

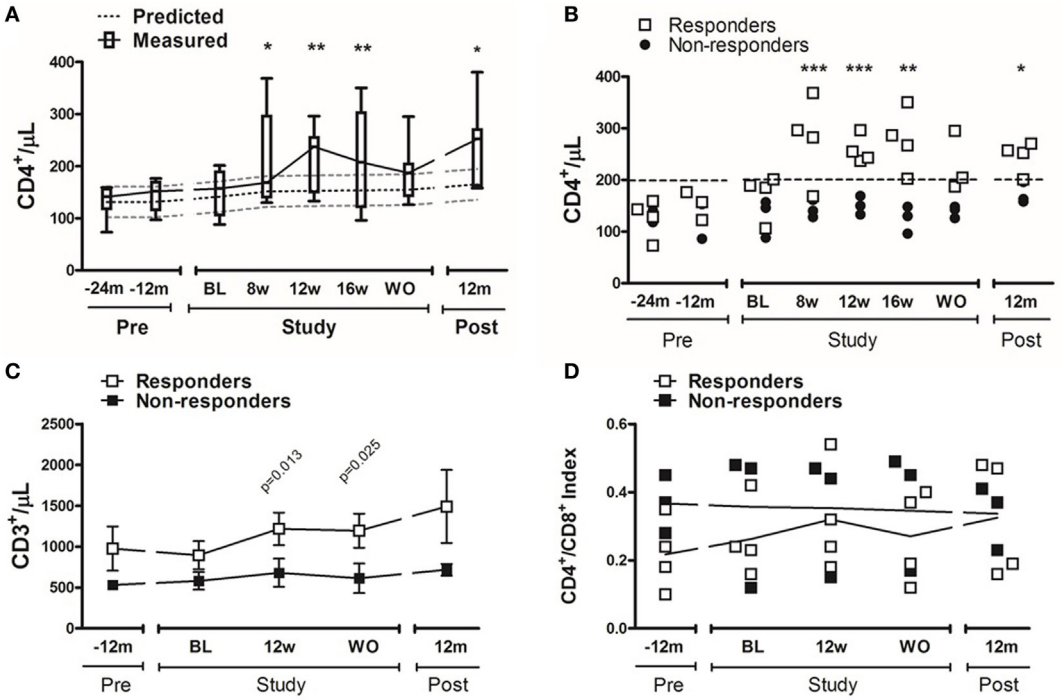

FIGURE 2 | CD4+ cell increase before, during, and after pyridostigmine (PDG). Total CD4+ cell counts were determined by flow cytometry as CD3+, CD4+, and $CD8^{neg}$ cells. **(A)** The solid line shows the dynamic increase of total CD4+ T-cells [median (p25–p75)] during PDG administration; for comparison, the broken lines depict predicted increase in circulating CD4+ cells of 35 cells/mL/year (4). **(B)** A subgroup of patients showed a significant, rapid, and persistent elevation in CD4+ cells, and this increase persisted after washout, and 1-year after PDG (each symbol represents and individual value). **(C)** Total T-cells were determined by flow cytometry as CD3+ cells (mean ± SD). **(D)** The ratio of CD4+ and CD8+ cells was determined by gating from total (CD3+) T-cells (each symbol represents and individual value). $n = 7$ for panel **(A)**; $n = 3$–4 subjects/group for panels **(B–D)**. *$p \leq 0.05$; **$p \leq 0.01$ vs. baseline.

CD4+ T-cell counts decreased to similar levels to those observed before inclusion. One year after the discontinuation of PDG, CD4+ T-cell counts were significantly elevated in comparison with baseline (baseline = 153.2 ± 43.1 vs. 1-year = 213.9 ± 45.9 cells/μL; $p = 0.02$) (**Figure 2A**).

We then performed a *post hoc* analysis, separating participants dichotomically according to CD4+ T-cell response: for that, we defined patients whose CD4+ T-cells reached 200 cells/μL as *responders*, and those who failed to achieve 200 cells/μL as *non-responders*. Responders separated from the non-responders early after they started taking PDG (**Figure 2B**). Moreover, among responders, the CD4+ T-cell counts remained significantly increased for the duration of intervention, returned to baseline levels after discontinuation of PDG. We observed a trend for higher baseline levels of total T-cells (CD3+) among responders than non-responders (895.5 ± 345.0 vs. 581.0 ± 186.8 cells/μL, respectively; $p = 0.11$); by week-12, responders had significantly higher total T-cell counts than non-responders (1,217.0 ± 396.7 vs. 681.0 ± 297.2 cells/μL, respectively; $p = 0.05$) (**Figure 2C**). The CD4+/CD8+ index remained stable during the study period for all subjects, suggesting that the increase in CD4+ cells was due to a global increase in T-cells rather than to an effect on CD4+ cells (**Figure 2D**).

While no individual characteristic predicted response, non-responders were older, had been diagnosed at an older age, had been untreated for HIV infection for twice as long after being diagnosed, and had lower mean CD4+ T-cells at baseline, suggesting that the lack of response might be due to age-related decreased thymic output (**Table 1**).

DISCUSSION

In this proof-of-concept paper, we present data suggesting that add-on therapy with the ACh-esterase inhibitor PDG improves the number of circulating CD4+ T-cells in HIV-infected, immunological non-responder patients (INRs).

In HIV infection, initiation of cART leads to reduced viremia, increased CD4+ T-cell counts, normalization of the CD4+/CD8+ ratio, and reduction in morbidity and mortality (3). Most patients using cART respond by effectively shutting down viremia and increasing CD4+ T-cell counts. Around 20% of patients starting cART with advanced disease show an insufficient increase of CD4+ cells even after adequate suppression of viral replication (9).

A CD4+ count below 200 cells/μL implies severe immunosuppression and precludes the discontinuation of primary prophylaxis against opportunistic infections (e.g., *Pneumocystis* pneumonia or *Toxoplasma gondii* encephalitis). The clinical consequences of being an INR are dire, because the severe CD4+ lymphopenia increases dramatically the risk of developing opportunistic

infections and malignant disease (10, 11). Further, low $CD4^+$ T-cell counts are associated with faster disease progression and mortality (5, 12, 13).

The mechanisms underlying $CD4^+$ T-cell lymphopenia in patients infected with HIV are incompletely understood. Nonetheless, persistent immune activation, observed even in patients with suppressed viral replication, is considered a critical factor for $CD4^+$ T-cell depletion (14). A number of pharmacological strategies, including hydroxychloroquine, statins, cyclosporine A, mycophenolic acid, and rapamycin, have unsuccessfully tried to improve the cellular response in HIV-infected patients (14–16). In a previous report, we showed that PDG is able to modulate T-cell activation—as reflected by cytokine production and cell proliferation—in patients with HIV infection (8). Based on those findings, we hypothesized that T-cell modulation through enhanced Ach availability could improve $CD4^+$ T-cell counts in patients with HIV infection that had failed to accomplish adequate immune reconstitution.

In this study, more than half of the patients treated with PDG experienced an increase in $CD4^+$ T-cell numbers of sufficient magnitude to allow them to cross the clinically relevant threshold of 200 cells/μL. Moreover, although the effect was greatest during exposure to the drug, it showed to be long lasting and a year after the discontinuation of the experimental therapy, the responders maintained $CD4^+$ T-cell counts higher than 200 cells/μL.

The nervous system senses cytokines and other inflammatory signals and responds, through an anti-inflammatory reflex, *via* the vagus nerve. The efferent branch of this system is known as the CAP. Accordingly, electrical stimulation of the vagus nerve, or administration of cholinergic agonists, inhibit the inflammatory response and lower the mortality of experimental endotoxemia and other cytokine-mediated inflammation by inducing or facilitating the CAP (17). Likewise, activation of the CAP during systemic inflammation downregulates the production and release of inflammatory cytokines (18).

T-cells are affected by the CAP and accordingly, they express all the critical components for ACh signaling, including nicotinic and muscarinic receptors, as well as the enzymatic machinery needed for ACh synthesis (19–21). ACh can activate or suppress T-cells, depending on the kinetics of its stimulation. Brief stimulation of nicotinic receptors on T-cells results in Ca^{2+} signaling and cell activation, while sustained stimulation results in downregulation of T-cell activation (19, 22).

Pyridostigmine, a reversible ACh-esterase inhibitor, has been in use since the mid-1950s for symptomatic treatment of myasthenia gravis (23, 24) and as prophylactic agent against biological warfare involving neurotoxic agents (25, 26). By inhibiting ACh esterase, PDG increases the half-life of endogenous ACh. Because of its hydrophilic nature, its penetrance to the central nervous system is limited and thus it is considered the standard of care for myasthenia gravis and for civilians and soldiers at risk of exposure to nerve agents (e.g., sarin) (27, 28). The data presented here demonstrate that in principle, enhancement of ACh availability may have a clinically relevant effect in increasing $CD4^+$ T-cell counts in a subset of INR patients with HIV infection.

As a pilot study, our work has many limitations, including the lack of a control group, a small sample size consisting only of male participants, and the use of only one dose of PDG. The choice of the PDG dose (90 mg/day) was based on previous reports finding less than 0.1% of serious side effects among 41,650 healthy soldiers taking PDG as prophylaxis against biological warfare (25). It is important to note, however, that as a proof-of-concept study, the present work was not designed to evaluate different doses. The optimal dose of PDG will have to be determined in future studies. Regarding gender imbalance, we invited all participants fulfilling our inclusion criteria, but only seven male patients signed informed consent. Interestingly, about 85% of HIV-infected individuals in Mexico are male; for reasons that are unrelated to HIV status (e.g., child rearing), about 95% of HIV-positive participants in clinical trials are male (29). Due to our limited sample size, we were not able to identify factors associated with response to PDG. Finally, we did not assess mechanism of response. All those questions will be addressed in an ongoing placebo-controlled trial.

In conclusion, our data suggest that add-on therapy with the ACh inhibitor PDG may improve the number of circulating $CD4^+$ T-cells in HIV-infected, immunological non-responder patients.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the institutional Ethics in Human Research Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics in Human Research Committee.

AUTHOR CONTRIBUTIONS

SIVF, JCC, and JSM designed the study, analyzed and interpreted data. SIVF, PFBZ, and JCC wrote the manuscript. PFBZ analyzed raw data and performed statistical analysis. CARO, BCD, JAV, and CCB helped collect and interpret data. All authors approved the final version of the manuscript.

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REVIEW
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Cholinergic Modulation of Type 2 Immune Responses

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In recent years, the bidirectional relationship between the nervous and immune system has become increasingly clear, and its role in both homeostasis and inflammation has been well documented over the years. Since the introduction of the cholinergic anti-inflammatory pathway, there has been an increased interest in parasympathetic regulation of both innate and adaptive immune responses, including T helper 2 responses. Increasing evidence has been emerging suggesting a role for the parasympathetic nervous system in the pathophysiology of allergic diseases, including allergic rhinitis, asthma, food allergy, and atopic dermatitis. In this review, we will highlight the role of cholinergic modulation by both nicotinic and muscarinic receptors in several key aspects of the allergic inflammatory response, including barrier function, innate and adaptive immune responses, and effector cells responses. A better understanding of these cholinergic processes mediating key aspects of type 2 immune disorders might lead to novel therapeutic approaches to treat allergic diseases.

Keywords: parasympathetic nervous system, allergic inflammation, type 2 immunity, cholinergic anti-inflammatory pathway, neuroimmune interactions, vagus nerve

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INTRODUCTION

Evidence is accumulating that extrinsic nerves intensely and bidirectionally communicate with the immune system. Indeed, in 2000, Tracey and coworkers introduced the novel concept of the "inflammatory reflex," where afferent vagal fibers sense peripheral inflammation and inform the brain on the inflammatory status to subsequently trigger vagal efferent fibers to dampen the inflammatory response (1). The cross talk between the vagus nerve and the immune system has been largely attributed to dampening of the innate immune system, in particular by reduction of cytokine release by splenic macrophages (2, 3). Since then, vagus nerve stimulation (VNS) has been evaluated in several inflammatory models characterized by not only activation of the innate immune system but also the adaptive immune system, particularly Th1 or Th17 immune responses (4, 5). Clinical observations, however, indirectly suggest that cholinergic immune modulation may also be of interest in atopic disorders characterized by T helper 2 (Th2) responses. Nicotine, a major constituent of cigarette smoke and a ligand for nicotinic acetylcholine receptors (nAChRs), has been implicated in anti-inflammatory effects seen in correlation with smoking. However, a rather complex relationship exists between smoking and the incidence of allergic diseases; on the one hand, cigarette smoke has been reported to cause impaired lung function, consequently aggravating asthma symptoms, and additionally also increase the risk to develop atopy, asthma, and food allergy (6–10). Oppositely, other reports indicated an inverse correlation between smoking and allergic

disease incidence. Epidemiological studies demonstrated that the occurrence of asthma could be higher in former smokers, compared with active smokers (11–13). In addition, other studies have observed a negative association between smoking and the development of allergic sensitization (14, 15). In accordance with this, in a recent study Tilp et al. demonstrated that cigarette smoke reduces the allergen-induced Th2 response in the lung and the development of airway hyperreactivity (AHR) in a severe model of asthma in mice (16). Similarly, the adverse effects of excessive psychological stress on the incidence and severity of allergic disorders have been acknowledged for long time (17). Interestingly, anxiety and depression have been associated with a decrease in vagal tone, leading to the exploration of VNS as a treatment for depression (18–21). The possibility exists that the decrease in vagal tone leads to a reduction in cholinergic modulation promoting allergic inflammation. This theory is supported by a study demonstrating that impaired parasympathetic function increased susceptibility to inflammatory bowel disease in a mouse model of depression (22). Furthermore, acetylcholinesterase (AChE) inhibitors, which have been successfully used in animal models to activate the cholinergic anti-inflammatory pathway, are widely used in the clinic to treat Alzheimer's disease (AD) (23–26). Curiously, AD patients treated with the AChE inhibitor, donepezil, showed increased serum levels of interleukin (IL)-4 and MCP-1, suggesting a possible shift toward a Th2 immune response (27). In addition, the effect of organophosphate compounds (OPCs), which are used as pesticides and are known AChE inhibitors, has been tested in allergic animal models. However, contrasting results have been obtained, whereas some studies mention an aggravation of allergic immune responses, other studies report an overall immunosuppressive effect (28–31). This discrepancy might be attributed to the timing of exposure, as a recent study showed that exposure to OPCs before allergen sensitization leads to worsening of allergic inflammation, while coinstantaneous exposure leads to suppression (31). Over the years, the importance of cholinergic modulation in inflammatory conditions has become increasingly clear. Although the role of parasympathetic regulation in allergic disorders has not been investigated extensively, more evidence is emerging supporting a role for cholinergic modulation in Th2-mediated disorders. Therefore, in this review, we aim to provide an overview of recent discoveries and its implications in relation to this research area.

PATHOPHYSIOLOGY OF ALLERGIC INFLAMMATION

At mucosal surfaces, the epithelium is crucial for maintaining a physical barrier and protect against environmental insults and allergens. In addition, it forms a functional barrier allowing communication between the internal and external environment. A whole array of different immune cells exists within the mucosal barrier, constantly surveying the environment and ready to provide innate and adaptive immunity. Many allergic diseases typically coincide with disorders of the epithelial barrier, which is associated with loss of epithelial differentiation, reduced junctional integrity, and impaired innate defense. For a long time, barrier dysfunction was seen as a consequence of the aberrant immunological response toward allergens. Recent evidence, however, suggests that barrier dysfunction also may act as an important initiator and player in the pathophysiology of allergic inflammation. The barrier can be disrupted by a number of factors arising from the external environment, such as proteases, toxins, or injury, but also endogenous factors such as hormones, diet, and circadian clock could contribute. Disruption of the barrier might result in increased antigen uptake and can drive predisposition to allergic sensitization (32, 33). On exposure, allergens are sensed by epithelial cells, which in turn lead to the rapid release of epithelial cytokines, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (34). These epithelial cytokines are able to induce expression and release of type 2 cytokines by type 2 innate lymphoid cells (ILC2s). This creates an environment that promotes the generation of allergen-specific Th2 cells, a process that is mainly driven by antigen presenting cells, primarily dendritic cells (DCs). DCs are responsible for taking up, processing and presenting allergens to naïve T cells. Th2 cells then play a central role, orchestrating different events of the inflammatory allergic response, mainly through the release of type 2 cytokines such as IL-4, IL-5, IL-9, and IL-13. These events include the attraction of effector cells such as eosinophils, mast cells, and basophils to the site of inflammation. In addition, B-cell class switching will be promoted under the influence of IL-4 and IL-13, leading to an increased production of allergen-specific immunoglobulin E (IgE). Upon allergen reexposure, IgE is able to bind FcεRIα on the surface of mast cells and basophils leading to their activation. This cascade of events can occur at different sites throughout the body and represent the underlying mechanism of disorders such as allergic rhinitis, asthma, food allergy, and atopic dermatitis (**Figure 1**) (35, 36).

THE CHOLINERGIC NERVOUS SYSTEM IN GUT, LUNG AND SKIN

Barrier surfaces such as the gastrointestinal (GI) tract, respiratory tract, and skin are densely populated by neurons and immune cells that constantly sense and respond to environmental challenges, including allergens. The peripheral nervous system (PNS) consists of the somatic nervous system and the autonomic nervous system. The latter can be further subdivided into the parasympathetic, sympathetic, and enteric nervous system (ENS). The different neurons of the PNS have been found to communicate with the immune system through the release of neuromediators from their nerve terminals. The parasympathetic nervous system primarily uses the neurotransmitter acetylcholine (ACh). As in this review, the focus will be on cholinergic modulation of the immune response, we will first describe the parasympathetic innervation and cholinergic input at the different epithelial barriers typically involved in allergic conditions.

The gut is densely innervated by the autonomic nervous system, consisting of the extrinsic innervation and the ENS, located within the intestine. The vagus nerve, providing a bidirectional

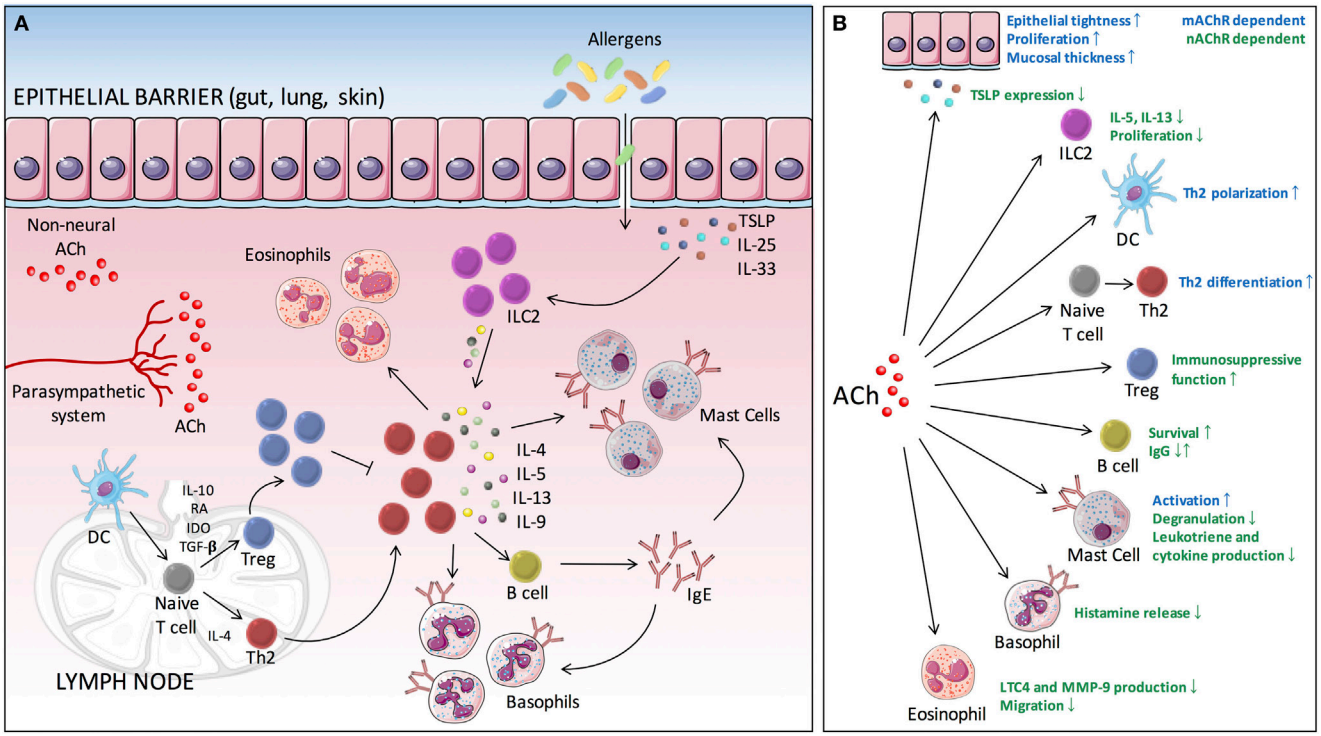

FIGURE 1 | Cholinergic modulation of type 2-mediated inflammation. **(A)** Allergen exposure evokes the rapid release of epithelial cytokines (IL-25, IL-33, and TSLP), which in turn drives the production of type 2 cytokines by ILC2s. This creates an environment that further facilitates DCs to promote the differentiation of naive T cells toward Th2 cells. These Th2 cells will then orchestrate different events of the inflammatory allergic response, through the release of type 2 cytokines (IL-4, IL-5, IL-9, and IL-13), including the attraction of effector cells (eosinophils, mast cells, and basophils) to the site of inflammation. In addition, B-cell class switching is promoted, leading to the production of allergen-specific IgE. Binding of antigen to IgE bound on mast cells and basophils leads to IgE cross-linking and subsequent activation. **(B)** ACh is able to modulate several key processes of the allergic inflammatory response *via* muscarinic and nicotinic receptors. ACh, acetylcholine; TSLP, thymic stromal lymphopoietin; DC, dendritic cell; ILC2, type 2 innate lymphoid cell; Th, T helper cell; IgE, immunoglobulin E; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; IL, interleukin; Th2, T helper 2.

connection between the brain and the gut, represents the main extrinsic parasympathetic nerve in the GI tract, where it mainly controls secretion, vascularization, and gastrointestinal motility. Preganglionic efferent vagal nerve fibers extensively innervate the GI tract, displaying the highest density in the stomach and further decreasing in the small bowel and colon, and establishing connections with postganglionic neurons primarily located in the myenteric plexus (37, 38). However, as vagal efferents only synapse with cholinergic enteric neurons in the myenteric plexus, it is likely that they affect mucosal immune responses indirectly through activation of cholinergic ENS neurons releasing ACh (39).

In the lung, the parasympathetic nervous system plays a prominent role in the control of airway smooth muscle tone. ACh released from postganglionic neurons induces bronchoconstriction, mucus secretion, and bronchial vasodilation, primarily mediated *via* binding on muscarinic receptor M3 (40, 41). For this reason, anticholinergic and muscarinic antagonists have been used to treat bronchoconstriction in asthma. The prominent role of the parasympathetic nervous system in the pathophysiology of asthma makes it challenging to investigate its role in the modulation of the immune response.

In contrast to the GI and the respiratory tract, the skin is devoid of parasympathetic innervation (41). This might question a role for cholinergic modulation of immune responses in the skin and in diseases, such as atopic dermatitis. However, the skin contains other sources of ACh, in particular keratinocytes (42), but in fact almost every cell, including epithelial, endothelial, and immune cells can produce ACh. Hence, this so-called "non-neural cholinergic system" might not only be of relevance in the skin but also in the gut and lung (43).

CHOLINERGIC MODULATION OF BARRIER FUNCTION

Improving epithelial barrier function could result into a decreased access of allergens, limiting the subsequent type 2 inflammatory response. Although there is no direct evidence for cholinergic modulation of epithelial barrier function in allergic disorders, some studies do suggest a role for ACh in modulating barrier integrity. ACh was shown to play a role in the regulation of epithelial tightness in pig colon cultures. Incubation with carbachol resulted into an increased transepithelial electrical

resistance, an effect that was inhibited by atropine, suggesting involvement of muscarinic acetylcholine receptors (mAChRs) (44). In addition, muscarinic agonists where shown to stimulate epithelial cell proliferation, increasing mucosal thickness in the intestine. Moreover, in several inflammatory conditions, cholinergic modulation was seen to protect barrier integrity due to improved tight junction protein expression (45–48). However, this effect is probably indirectly regulated by the downregulation of pro-inflammatory cytokines. Although the cholinergic modulation of barrier function in type 2-mediated diseases has been relatively unexplored so far, it might hold yet undiscovered potential toward therapeutic interventions.

The epithelium should not be considered as merely a physical barrier controlling the uptake and transport of antigens; in addition, it should be seen as an active contributor to the mucosal environment helping to shape local immune reactions. Epithelial derived cytokines IL-25, IL-33, and TSLP have been shown to play a role in the initiation of type 2 allergic responses. Preventing the expression and release of epithelial cytokines might be sufficient to prevent the subsequent Th2 immune response. Evidence that cholinergic modulation might influence this process was recently provided by a study showing inhibition of TSLP expression both *in vitro* and *in vivo* by nicotine. This effect was abolished by pretreatment with α7 nAChR antagonists, showing the involvement of α7 nAChR (49).

Experimental and clinical data have supported a role for type 2 cytokines in the regulation of intestinal barrier function. IL-4 and IL-13 have been shown to increase intestinal permeability, an effect that is mediated *via* phosphoinositide 3-kinases (50–53). In addition, allergic effector cells such as mast cells and eosinophils release powerful inflammatory mediators, such as proteases and cytokines, further contributing to barrier dysfunction (52, 54–56). Cholinergic modulation of immune cells involved in the allergic inflammatory response might therefore, in addition to direct effects that will be described in detail below, also contribute to effects on barrier function.

CHOLINERGIC MODULATION OF THE TYPE 2 INNATE IMMUNE RESPONSE

The innate immune system plays an important role in the initiation of subsequent adaptive immune response. Key players of type 2 innate immunity include recently described ILC2s that produce cytokines promoting Th2 differentiation by DCs, which are considered as a crucial link between innate and adaptive immunity.

Interestingly, a recent study showed that ILC2 might be modulated by cholinergic signals. Indeed, α7 nAChR expression has been shown on ILC2, whereas *in vitro* incubation of pulmonary ILC2s with GTS-21, a specific α7 nAChR agonist suppressed production of IL-5 and IL-13 cytokines, proliferation, and GATA3 expression. In addition, GTS-21 treatment ameliorated ILC2 induced AHR. Similar observations were made in a humanized ILC2 AHR mice model (57). These findings suggest that cholinergic modulation of ILC2s *via* the α7 nAChR is a potential target for therapeutic intervention in type 2-mediated disorders.

In contrast to the well described anti-inflammatory effect of ACh on macrophages (39, 58–60), the effect of cholinergic modulation on DCs has been far less studied. Aicher et al. demonstrated the expression of α7 nAChR on human monocyte-derived DCs, which was upregulated in response to its ligand nicotine (61). In addition, murine bone marrow-derived DCs expressed mRNAs encoding the five mAChR subtypes (M1–M5) and the α2, α5, α6, α7, α10, and β2 nAChR subunits (62). Earlier reports suggested an anti-inflammatory effect of nicotine reducing endocytosis, phagocytosis, the production of pro-inflammatory cytokines IL-12, IL-1β, and TNF-α and the ability to induce a Th1 response (63). By contrast, other reports stated that nicotine activated DC function, enhancing endocytosis, stimulation of T cell proliferation, and the production of IL-12 (61, 64). These opposing results might be due to differences in maturation and activation state. More recently, Gori et al. demonstrated a role for ACh in polarizing DCs toward a Th2-promoting phenotype. Treatment of DC with ACh stimulated the expression of the Th2-promoter OX40L, the production of Th2-chemokines CCL22 and CCL17, and the synthesis of IL-4, IL-5, and IL-13 by T cells (65). This study was in line with earlier findings by Liu et al. showing that the muscarinic receptor agonist, methacholine, increased OX40L in DCs (66). These data suggest an involvement of mAChRs in the Th2 polarizing effect of ACh on DCs.

CHOLINERGIC MODULATION OF THE TYPE 2 ADAPTIVE IMMUNE RESPONSE

Given that DCs form a bridge between the innate and adaptive immune responses, cholinergic modulation of these cells as described earlier will definitely influence consequent adaptive immune responses. However, both T and B lymphocytes are also directly modulated by ACh, as several nAChR and mAChRs subtypes have been reported in both cell types (67–70).

As for other immune cells, also T cells show a high degree of plasticity with respect to their cholinergic receptor network. Depending on their activation state, differentiation state or even environmental cues, T cells will express a different repertoire of ACh receptors (71). So far, especially α7 nAChR has been investigated in the context of the cholinergic anti-inflammatory pathway (1). Although the expression of the α7 nAChR subunit has been demonstrated in activated CD4 T cells, surprisingly Th1, Th2, or Th17 cells do not express detectable levels of this receptor (71). In addition, Although vagotomy has been shown to increase T cell proliferation and production of pro-inflammatory cytokines, this effect is not attenuated by selective α7 nAChR agonists, suggesting the involvement of other subunits such as α4, β2, and α5 (69, 72, 73). Other studies, however, do highlight the importance of the α7 nAChR in the anti-inflammatory effect of nicotine. Interestingly, a study by Galitovskiy et al. showed that α7 nAChR mRNA expression in colonic CD4 T cells was highly dependent on the inflammatory milieu. IL-4 was shown to upregulate α7 nAChR gene expression, whereas it was reduced by IL-12, possibly explaining the dualistic effect of nicotine in ulcerative colitis (Th2) versus Crohn's disease (Th1/Th17) (74). This finding is especially

of interest in allergic disorders, which are characterized by a strong type 2 inflammatory environment. In addition, a more recent study proposed α7 nAChR as a critical regulator for the immunosuppressive function of Tregs, mainly due to upregulation of immunoregulatory molecules CTLA-4 and FoxP3, and decreasing IL-2 levels (75). Considering the crucial role of Tregs in immune homeostasis and their capacity to suppress Th2 responses to allergens and other type 2 effector cells, novel ways to promote Treg cell stability and function, are being explored extensively in the treatment for allergic diseases. Whereas there has been an increasing appreciation for nicotinic receptors in the modulation of inflammation and homeostasis, muscarinic receptors have received much less attention so far. Of note, several studies have highlighted the importance of the M3 receptor in the generation of type 2 immune responses. Stimulation of mAChRs on naïve CD4 T cells promotes Th2 and Th17 lineages, while blockade of mAChR will lead to Th1 differentiation (71). Moreover, two recent studies demonstrated the importance of M3 receptor activity in the generation of Th2 responses in an enteric nematode infection model and an allergic airway inflammation model (76, 77). These studies suggest that the M3 receptor opposes the effect of α7 nAChR receptor activation (77). It should be emphasized though that the range of doses that activates each receptor is some log units apart, questioning if this interplay is indeed of physiological relevance. So far, most findings concerning cholinergic modulation of T cells are based on *in vitro* studies, hardly representing the complex nature of the inflammatory environment. Therefore, to better understand the functional consequences of cholinergic activation on T cells *in vivo*, further studies in experimental models are needed.

As mentioned earlier, functional expression of nAChR subunits and mAChRs subtypes has also been demonstrated in B cells (67–70). Information on the functional implications of cholinergic receptor activation is, however, rather limited. Nicotine affects the survival of B lymphocyte precursors during differentiation, an effect that was dependent on both α7 and β2 subunits (78). In addition, effects on antibody responses have been reported. A study by Skok et al. described a role α4β2 and α7 nAChRs in the formation of the pre-immune status and the regulation of antibody response, possibly by affecting CD40 expression and/or signaling. Mice lacking α4, β2, or α7 nAChR subunits exhibited lower levels of IgG antibodies (79, 80). By contrast, Fuji et al. reported higher levels of IgG1 antibodies in α7 nAChR knockout mice (81). The reason for this discrepancy is still unclear. However, both studies reported increased levels of IgG antibodies after immunization in the absence of α4, β2, or α7 nAChR subunits. Of note, while the effect on antibody response could be directly regulated *via* cholinergic receptors on B cells, it could also be attributed to cholinergic inhibition of pro-inflammatory cytokines by other immune cells, indirectly leading to the suppression of IgG antibodies. Also in humans, effects on antibody responses have been observed. When comparing smokers with non-smokers, the former were shown to exhibit lower levels of all serum immunoglobulins, except for IgE (82, 83). Unfortunately, cholinergic modulation of B cells in inflammatory conditions has been relatively unexplored until now, so its role in allergic inflammation remains unclear.

CHOLINERGIC MODULATION OF ALLERGIC EFFECTOR CELLS

Mast cells, basophils, and eosinophils are considered as central effector cells in allergic inflammation. Their activation and subsequent release of granule stored mediators leads to many of the characteristic pathophysiological features observed in allergic responses. Ways to modulate these effector cells and to suppress their activation hold promise for the development of therapeutics for allergic diseases. All three types of granulocytes are shown to express cholinergic receptors, and are thus prone to cholinergic modulation.

Both muscarinic and nicotinic cholinergic receptors have been identified on mast cells (84–86). Moreover, mast cell–nerve associations have been shown in both small intestine and colon (87–89). Vagotomy in rats resulted into a decrease of mast cells in the jejunal mucosa (90). VNS on the other hand was shown to elevate histamine levels in the jejunal wall. To what extent this effect was due to increased synthesis or decreased release of histamine remains unclear (91). Thus far, studies describing the functional effects of cholinergic mast cell modulation have been rather conflicting. Early studies reported activation rather than inhibition of mast cell degranulation by ACh *via* muscarinic receptors (92–94). Other studies, however, failed to show an effect of ACh on mast cell degranulation (95, 96). This discrepancy was addressed by Masini et al., demonstrating that incubation with IgE increased the sensitivity of mast cells to ACh and induced a more homogeneous response (94). Nicotinic receptors on the other hand seem to mediate an anti-inflammatory effect. Expression of nicotinic receptor subunits α4, α7, and β2 was found in mouse bone marrow-derived mast cells. In addition, nicotine was shown to inhibit antigen-IgE-induced degranulation of mast cells in a dose dependent manner, an effect that was mimicked by the α7 nAChR subunit agonist, GTS-21. α-Bungarotoxin, a specific α7 antagonist, significantly inhibited the suppressive effect of GTS-21, further confirming the observed effect is α7 nAChR dependent (85). In line, α7, α9, and α10 receptor subunits are expressed in a rat mast cell/basophil cell line, RBL-2H3, in which nicotine induces suppression. Although nicotine did not inhibit degranulation, it suppressed late phase FcεRI-induced leukotriene and cytokine production, an effect that could be abrogated by siRNA mediated ablation of α7, α9, or α10 nAChRs (97). Interestingly, a recent study by Yamamoto et al. demonstrated a potential therapeutic effect for the cholinergic anti-inflammatory pathway in an experimental murine model of food allergy. Vagal stimulation by using 2-deoxy-D-glucose, a central vagal stimulant, ameliorated the development of food allergy, and was reversed by the nAChR antagonist, hexamethonium. Moreover, similar effects were obtained with nicotine and GTS-21, indicating the involvement of α7 nAChR. It was suggested that the amelioration of allergic symptoms where due to the suppression of mucosal mast cells; however, no direct evidence was provided. The possible involvement of other immune cells can thus not be excluded (89).

Basophils share many similarities with mast cells and are often considered as their circulating counterparts. However, the cholinergic modulation of basophils has been far less investigated

compared with mast cells. So far, the earlier mentioned rat mast cell/basophil cell line, RBL-2H3 was shown to express α7, α9, and α10 nAChR receptor subunits (97). In addition, fluorescently labeled α-bungarotoxin was shown to bind to the surface of a human basophil cell line, and more recently the presence of nAChR α4, α7, and α1/α3/α5 subunits was shown in human blood basophils by flow cytometry (98, 99). In parallel with the observations in mast cells, Thompson-Cree et al. showed that nicotine agonists were able to inhibit IgE-induced histamine release from human peripheral blood basophils (100). In addition, a recent study showed similar *in vitro* and *in vivo* inhibition of human blood basophils from mild allergic asthma patients using ASM-024, a novel compound with nicotinic and muscarinic effects (99). The anti-inflammatory effect of ASM-024 was moreover confirmed in a murine model of allergic airway inflammation (101).

Eosinophil–cholinergic nerve interactions have been documented in several tissue compartments under inflammatory conditions, including lung, intestine, nose, and skin (102–108). The interaction between eosinophils and nerves seems to be mediated *via* adhesion molecules ICAM-1 and VCAM-1 and has been correlated with cholinergic nerve remodeling (103, 109). Eosinophil adhesion results in degranulation leading to the release of mediators such as major basic protein (MBP) and eosinophil peroxidase (EPO) (103). Binding of MBP and EPO leads to increased release of ACh due to upregulation of choline acetyltransferase and vesicular ACh transporter gene expression, and in addition due to antagonistic effect on inhibitory M2 receptors (110–112). Recently, modulation of cholinergic nerves by eosinophils has become increasingly clear and might contribute to pathological processes, such as smooth muscle contraction and increased mucus production, observed in allergic conditions. However, a possible anti-inflammatory effect of ACh on eosinophils should not be discarded. Expression of α3, α4, and α7 nAChR subunit transcripts and protein has been demonstrated in human blood cells. Moreover, it was shown that stimulation of these cells *in vitro* with DMPP, a nicotinic agonist, downregulated eosinophil function. DMPP inhibited LTC4 production, eosinophil migration, MMP-9 production, and intracellular calcium mobilization (113). In addition, DMPP was used earlier in an *in vivo* model of asthma, where it prevented airway hyperresponsiveness and reduced the number of lymphocytes and eosinophils. The obtained results could be in part attributed to the observed effects on eosinophil function; however, additional effects on other immune cells cannot be excluded (113, 114).

CONCLUSION

In recent years substantial advances have been made with respect to understanding the complex interactions between the nervous system and the immune system. The findings described in this review have highlighted a so far underappreciated role for the parasympathetic nervous system and cholinergic modulation in controlling type 2 adaptive immune responses. Several key players of the pathophysiological mechanism underlying allergic disorders are subject to cholinergic modulation (**Figure 1**) and could thus be exploited in the search for new therapeutic approaches to treat allergies. However, it should be noted that neuroimmune modulation is not only limited to the parasympathetic nervous system. Different neurotransmitters, originating from the parasympathetic, sympathetic, and ENS, are able to influence each other. For instance, neurotransmitters of the parasympathetic and the sympathetic nervous system are often found to exert antagonistic effects. In addition, immune cells render an enormous plasticity what expression of neurotransmitter receptors is concerned. A number of factors including the local microenvironment, which will differ in health versus disease, the maturation or activation state of cells, will influence receptor expression. Predicting the outcome of therapeutic interventions using specific receptor agonists and antagonists will therefore be challenging. A deeper understanding of how the different branches of the nervous system interact with each other and with the immune system, in both homeostasis and inflammation, will hold considerable promise for the development of new and innovative therapeutic strategies for allergic disorders and other inflammatory conditions.

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REVIEW
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The Role of Brain-Reactive Autoantibodies in Brain Pathology and Cognitive Impairment

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Antibodies to different brain proteins have been recently found to be associated with an increasing number of different autoimmune diseases. They need to penetrate the blood–brain barrier (BBB) in order to bind antigens within the central nervous system (CNS). They can target either neuronal or non-neuronal antigen and result in damage either by themselves or in synergy with other inflammatory mediators. Antibodies can lead to acute brain pathology, which may be reversible; alternatively, they may trigger irreversible damage that persists even though the antibodies are no longer present. In this review, we will describe two different autoimmune conditions and the role of their antibodies in causing brain pathology. In systemic lupus erythematosus (SLE), patients can have double stranded DNA antibodies that cross react with the neuronal *N*-methyl-D-aspartate receptor (NMDAR), which have been recently linked to neurocognitive dysfunction. In neuromyelitis optica (NMO), antibodies to astrocytic aquaporin-4 (AQP4) are diagnostic of disease. There is emerging evidence that pathogenic T cells also play an important role for the disease pathogenesis in NMO since they infiltrate in the CNS. In order to enable appropriate and less invasive treatment for antibody-mediated diseases, we need to understand the mechanisms of antibody-mediated pathology, the acute and chronic effects of antibody exposure, if the antibodies are produced intrathecally or systemically, their target antigen, and what triggers their production. Emerging data also show that *in utero* exposure to some brain-reactive antibodies, such as those found in SLE, can cause neurodevelopmental impairment since they can penetrate the embryonic BBB. If the antibody exposure occurs at a critical time of development, this can result in irreversible damage of the offspring that persists throughout adulthood.

Keywords: autoantibodies, brain, systemic lupus erythematosus, neuromyelitis optica, cognition, blood–brain barrier, maternal antibodies

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INTRODUCTION

Over the last several years, many different anti-brain antibodies have been associated with various autoimmune diseases (1). They can be classified as binding either neuronal or non-neuronal antigen and binding extracellular or intracellular antigen (2). Most importantly, the emerging questions are if they can be used for assessment of disease risk, severity, prognosis, and whether they contribute to brain pathology.

Some autoantibodies, such as those present in paraneoplastic disorders of the central nervous system (CNS) or in celiac disease, have been consistently reported to contribute to brain pathology and to cause neurological and cognitive impairment (3–7). For other anti-brain antibodies, such as those present in multiple sclerosis (MS) or narcolepsy, pathogenicity has not been established (8–10).

Given that some autoantibodies can be found also in a subset of healthy individuals (11), it is essential to determine if the antibodies can be used for diagnostic biomarkers of disease or if the autoantibodies are indeed pathogenic. In classic paraneoplastic disorders, where patients have antibodies against intracellular antigens, such as anti-Hu antibodies, it is believed that irreversible killing of neuronal cells is mediated by T cells (12), and the antibodies appear to be a secondary event. In contrast, in some diseases, such as neuromyelitis optica (NMO), the antibodies are pathogenic (13). When pathogenic antibodies enter the CNS, there are two possible outcomes. The pathological consequence of antibody exposure can be reversible. In limbic encephalitis associated with antibodies against cell surface receptors, such as antibodies against extracellular parts of the voltage-gated potassium channel (VGKC) complex, predominantly LGI1, or *N*-methyl-D-aspartate receptor (NMDAR) subunit GluN1, removal of pathogenic antibodies often results in complete remission of severe neuropsychiatric symptoms (12, 14). Alternatively, antibodies may trigger irreversible mechanisms that may continue even when antibody is no longer present in the brain. In patients with systemic lupus erythematosus (SLE) with cognitive dysfunction, pathology can be caused by acute antibody exposure to the NMDA receptor subunits GluN2A/GluN2B and proceed to chronic damage of surviving neurons even when brain-antibodies are no longer present (15).

While brain imaging continues to advance, it remains difficult to study human tissue in those brain diseases. Thus, animal models are needed to study transient and/or permanent tissue injury and to understand which pathology is the consequence of repeated exposure to antibodies and which pathology progresses even in the absence of continued exposure to brain-antibodies. Understanding the contribution of antibodies to disease pathogenesis is essential for the development of efficient and less invasive treatment options and for disease prevention.

INTRATHECAL IgG-SYNTHESIS OR SYSTEMIC IMMUNE REACTION

Brain-reactive antibodies can be produced intrathecally or can be passively transferred from the circulation to the CNS. For example, there is a growing body of evidence that autoantibodies in MS are produced intrathecally and that the presence of antibodies in the cerebrospinal fluid (CSF) is linked to oligoclonal antigen-specific B cells, which infiltrate the impaired blood–brain barrier (BBB) (16, 17). It has been suggested that the antibodies in the CSF of MS patients target ubiquitous intracellular antigens released as cellular debris (18), consequent to neuronal damage and, therefore, constitute a secondary process in disease progression. It may commonly be the case that intrathecal production of antibody is oligoclonal, as the only B cells to take up residence in the CNS may be those that have a B cell receptor for a brain antigen.

Antibodies can also reach the brain from the systemic circulation. Two main questions arise when a disease is caused by circulating anti-brain antibodies. The first question is what triggers their production. One possibility is that the antibodies are triggered by a bacterial or viral infection and cross-react with brain antigens that share structural similarities with the microbial target, a phenomenon defined as molecular mimicry, and was described for example in Sydenham's chorea (19). Lack of negative selection against CNS antigens as the repertoire of immunocompetent B cells is established might enable activation of B cells with cross-reactivity to brain. In paraneoplastic diseases, antibodies can be produced as a response to a tumor in a non-CNS site, such as lung or ovary, which expresses brain antigens (20).

The second question is how antibodies cross the BBB. BBB endothelial cells express tight junction proteins, which allow only strictly regulated transport into and out of the brain (21). To date, there is limited information available regarding the establishment of the BBB during development; however, there appears to be a time window during which antibodies can penetrate the fetal brain before an intact BBB is established (22). Anti-brain antibodies affecting the developing brain have been suggested to be the cause, for example, in a subset of Autism spectrum disorders, as well as lead to intellectual and cognitive impairments in children born to mothers with SLE (11, 23). In adulthood, certain insults to BBB integrity allow antibodies to penetrate CNS tissue. Different insults to the functionally established BBB lead to different regions of antibody penetration in the CNS. Depending on the location where antibodies gain access to the CNS tissue, various neurological symptoms might occur. Indeed in animal models, region-dependent effects are observed (24, 25). Moreover, circumventricular organs, such as the area postrema, the subfornical organ, and the vascular organ of lamina terminalis, lack tight junction proteins and might be an area for autoantibody entry in some autoimmune diseases (26). Some antibodies might even be able to cause BBB impairment by themselves. In NMO, antibodies to glucose-regulated protein 78 have been associated with BBB disruption (27). In experimental systems, most commonly, the BBB is breached by using either bacterial lipopolysaccharides (LPS) (25), epinephrine (24), and similar agents or by using different pathogenic CNS reactive T cells (28). These manipulations may all result in additional inflammation and make it more difficult to identify the antibody-mediated effect itself.

BRAIN ANTIBODIES AND THEIR PATHOGENICITY

Pathogenicity of brain-reactive antibodies depends on the accessibility of their target epitopes, the density of their presence in tissue and, if required, the presence of effector mechanisms in the brain in sufficient amounts (28). Antibodies from patients are often injected into rodent models and must result in a phenotype

similar to the one observed in the human disease to conclude that the antibodies themselves are pathogenic. However, not all patient-derived brain-reactive antibodies bind to rodent tissue; thus, a negative outcome regarding antibody pathogenicity in rodents has to be interpreted cautiously. This is the case in patients with different inflammatory CNS diseases associated with antibodies to myelin oligodendrocyte glycoprotein (MOG), where the majority of human anti-MOG antibodies do not recognize rodent MOG (29). In addition, pathogenicity of brain-reactive antibodies requires breach of the BBB when antibodies are injected systemically into the rodent blood stream. As stated above, insults to the BBB may add confounding factors to the study of antibody pathogenicity and will direct antibody penetration to certain brain regions, which may or may not be those brain regions most often targeted in patients. Alternatively, antibodies can be directly injected into the brain by stereotactic injection, which bypasses the need to breach the BBB. In order to investigate if the antibody results in cognitive impairment, a recognized and sometimes subtle consequence of antibody-mediated pathology, a battery of behavioral assays is performed using *in vivo* models. As more and more brain antibodies are discovered, we need to extend our *in vivo* studies to address whether pathological damage is caused by direct exposure to brain antibodies or if pathology persists even when the antibody is no longer present. Most studies have focused on the effect of acute antibody exposure; only a limited number of studies addressed a possible secondary stage of damage even when the antibody is no longer present in the brain. This secondary stage could be caused through inflammation caused by infiltrating T cells, microglial activation with secretion of proinflammatory cytokines.

For example, in a model of neurocognitive SLE, it is documented that anti-DNA/anti-NMDA receptor antibodies (DNRAbs) lead to persisting neuronal damage even after the antibodies are no longer present (15). It has been recently entertained that the surviving neurons are compromised as a secondary effect mediated by microglia (15). These extended *in vivo* studies are very important for future therapeutic targeting in disease, since removal of antibodies might prevent acute tissue damage, but may not address a subsequent disease phase.

Whereas *in vivo* models to study antibody-mediated brain disease in adults all require a BBB breach, pathogenicity of maternal anti-brain antibodies can be determined without BBB impairment since the fetal BBB allows penetration of antibodies for a period of time (23, 30). Thus, injection of antibodies into pregnant rodents or immunization of rodents with the antigen prior to pregnancy permits a subsequent investigation of the offspring for behavioral impairment and/or histological abnormality. Injecting antibodies into pregnant rodents enables the study of the effect of maternal antibody exposure at one particular time point, whereas immunization with the antigen results in exposure to maternal antibody throughout pregnancy. The binding of maternal anti-brain antibodies to embryonic brain will depend on the expression level of the antigen, which can vary from expression in the adult brain. Furthermore, some antigens exhibit distinct posttranslational modification in the embryonic brain; for example, there may be differences in glycosylation patterns of the antigen (31), which may affect the binding of the antibodies.

BRAIN ANTIBODIES AND THEIR MECHANISM OF ACTION

Following the proof of a pathogenic effect of brain-reactive autoantibodies, it is of central importance to investigate the pathogenic mechanism(s) in order to develop therapeutic interventions.

In some cases, preexisting inflammation may be required to reveal an antigenic epitope or antibody binding may lead to inflammation giving rise to inflammatory mediators that lead to pathology. Alternatively, complement-dependent cellular cytotoxicity (CDCC) or antibody-dependent cytotoxicity (ADCC) can cause target cell lysis, a possible mechanism of pathogenicity of some autoantibodies (32). Some antibodies can also result in cell death or dysfunction in the absence of inflammatory cell infiltration, CDCC, and ADCC, through altering cell signaling (32). Cell signaling alterations can also activate or impede cellular processes. Finally, antibodies can also cause internalization of membrane receptors, creating functional hypomorphs (2, 32).

The mechanisms of pathogenicity will determine the degree of recovery of brain function. Whereas CDCC and ADCC are more likely to result in irreversible tissue destruction, a pathogenic effect caused by internalization of membrane receptors can be reversed upon removal of antibodies, such as occurs in limbic encephalitis (6). In some autoimmune diseases, therefore, recovery of patients may be linked to the reestablishment of a functionally intact BBB, which prevents further antibody exposure in the CNS. In other autoimmune diseases, brain-antibodies result in a chronic condition, which may be due to constant antibody exposure or to pathology that is no longer dependent on the presence of antibodies (15). Similarly, *in utero* exposure to maternal brain antibodies can cause neurodevelopmental impairments in the offspring that persist throughout adulthood due to irreversible damage at a critical time of development (23, 30).

ILLUSTRATIVE EXAMPLES

In this review, we will describe two autoimmune conditions. First, SLE was discovered to be an autoimmune disease in the 1940s, but antibodies against defined neuronal antigens have been only recently described and linked to neurocognitive dysfunction (33–35). In SLE, pathology may be caused by acute exposure to brain-antibodies, but may persist even upon antibody removal due to irreversible damage and death of neurons and secondary pruning of healthy neurons (15). The role of microglial activation in this secondary disease phase remains to be investigated.

Second, NMO was initially described as a severe variant of MS but due to the discovery of anti-astrocytic antibodies and dramatically different responses to treatment (36, 37) NMO was segregated from MS and defined as a separate disease (38). In order to enable appropriate treatment, we need to understand the reversible and irreversible effects of aquaporin-4 (AQP4)-IgG-mediated tissue damage. In addition, it is important to understand the role of pathogenic T cells for disease initiation as well as for disease progression. Removal of antibodies or blocking of antibody-mediated mechanisms might not be sufficient to address possible disease progression even when the antibody is no longer present.

Neuron-Directed Antibodies in SLE

Systemic lupus erythematosus is a chronic autoimmune disease that is characterized by inflammation, pain, and tissue damage. SLE can affect any organ, including the brain (39). Since neuropsychiatric manifestations of SLE (NPSLE) are difficult to diagnose due to the diversity of clinical presentations, which include seizures, psychosis, cognitive dysfunction, and more (40), it is difficult to estimate the frequency of neuropsychiatric SLE (NPSLE). Many symptoms, such as headache or demyelination are not unique to NPSLE but can also be found in other autoimmune diseases. Studies claim that as few as 10% to as many as 90% of SLE patients suffer from neuropsychiatric symptoms (41). Cognitive impairment manifested as memory deficit is one of the most commonly observed symptoms in NPSLE patients (42), but is still poorly understood. It may be caused by a variety of mechanisms, both antibody and non-antibody mediated. Hypertension and accelerated atherosclerosis can also lead to cognitive impairment and confound the assessment of diseases-specific mechanisms.

To date, over 100 autoantibodies have been associated with SLE, of which, some associate with neuropsychiatric symptoms (43). Certain autoantibodies, such as anti-ribosomal P, anti-neurofilament, anti-endothelial, anti-Ro, or anti-Smith antibodies have been associated with neuropsychiatric manifestations other than cognitive impairment, whereas anti-neuronal, antiphospholipid, and anti-double stranded DNA (dsDNA) antibodies cross-reactive with the *N*-methyl-D-aspartate receptor (NMDAR) subunits GluN2A or GluN2B (anti-NR2) have been linked to neurocognitive impairment in SLE (44–48). Here, we describe in more detail the contribution of anti-dsDNA–NMDAR antibodies to cognitive impairment in SLE patients.

dsDNA–NMDAR CROSS-REACTIVE ANTIBODIES RESULT IN COGNITIVE IMPAIRMENT IN SLE

Anti-double stranded (ds) DNA antibodies are diagnostic of SLE. Previously, our group has shown that some SLE patients harbor anti-dsDNA antibody, which cross-react with a peptide sequence DWEYS present in the extracellular domain of the GluN2A and GluN2B subunits of the NMDAR. This cross-reactivity was first detected using the murine monoclonal anti-DNA antibody R4A. DNA-GluN2 cross-reactive antibodies (DNRAbs) bind to the extracellular part of GluN2 (49). DNRAbs can be detected either by ELISA or by a cell-based assay using human embryonic kidney (HEK) cells expressing the subunits GluN2A or GluN2B in combination with GluN1 (15, 50). They bind preferentially to the active configuration of the NMDAR and enhance the influx of calcium into the cell (51). They are found in approximately 40% of SLE patients (52). It remains to be investigated how the systemically produced DNRAbs gain access to the CNS. It has been suggested that they are able to breach the BBB by themselves (53), or other factors such as cytokines/chemokines or complement activation may be needed.

The pathogenicity of these antibodies was first demonstrated by injecting R4A into mouse brain, leading to apoptosis of neuronal cells. At lower concentrations, the antibody augments NMDAR-mediated synaptic potentials; at higher concentrations, it triggers mitochondrial stress and apoptosis through binding specifically to GluN2A-containing NMDARs (**Figure 1**). DNRAbs were eluted from the brain of a SLE patient and also caused neuronal apoptosis and cognitive impairment when injected into mice (25). Mice immunized with the DWEYS sequence multimerized on a polylysine backbone (termed MAP-DWEYS) develop DNRAbs, which cause loss of hippocampal neurons after LPS-induced compromise of BBB integrity (54). This occurs in the absence of inflammatory cell infiltration, CDCC, or ADCC. DNRAb-induced neuronal cell death results in cognitive dysfunction and spatial memory impairment associated with structural abnormalities in the surviving pyramidal neurons in the hippocampus (15). The change in spatial memory that occurred after LPS-facilitated DNRAb penetration into the hippocampus is accompanied by expansion in place field size of CA1 place cells in the hippocampus and shortened dendritic processes and spines of surviving hippocampal pyramidal cells (15). Remarkably, the functional and structural changes, which cause alterations in spatial cognition occur at a time when the antibodies are no longer present in the hippocampus and BBB integrity has been restored. Currently, we are investigating the role of microglial activation in the pathology. We believe that there is a two hit model in SLE. Our animal model showed that exposure of neurons to DNRAbs results in neuronal cell death. However, surviving neurons in the hippocampus show structural abnormalities, which are likely to be caused through secondary pruning of the surviving neurons by activated microglial cells (15). In contrast, removal of anti-brain antibodies in limbic encephalitis, which also target the NMDAR, mostly reverses disease symptoms, as these antibodies do not cause cell death.

In the animal model, neuronal cell death can be abolished through administration of the NMDAR antagonist memantine prior to BBB breach by LPS (24). Memantine has no effect on antibody binding, but blocks the triggering of NMDAR activation by DNRAbs.

Studies in patients show that NPSLE is associated with increased levels of GluN2A/GluN2B antibodies in the blood (55), and CSF titers of GluN2A/GluN2B antibodies correlate with the severity of NPSLE (56). Some studies have also associated cognitive impairments in NPSLE with the presence of anti-GluN2A/GluN2B antibodies (57, 58). Certainly, cognitive impairments in NPSLE will not be solely caused by those antibodies; other antibodies or cytokines likely also contribute to cognitive problems in NPSLE (55).

MATERNAL DNRAbs ARE NEUROTOXIC AND HAVE A GENDER-SPECIFIC EFFECT

During fetal development, pathogenic antibodies such as DNRAbs can penetrate the embryonic brain before the BBB is functionally established (59). Studies suggest an increased incidence of learning disabilities, fetal loss, and altered sex ratio in children of SLE mothers (60–63). It was, therefore, of interest to investigate the effect of DNRAbs on the fetal brain. We established a mouse

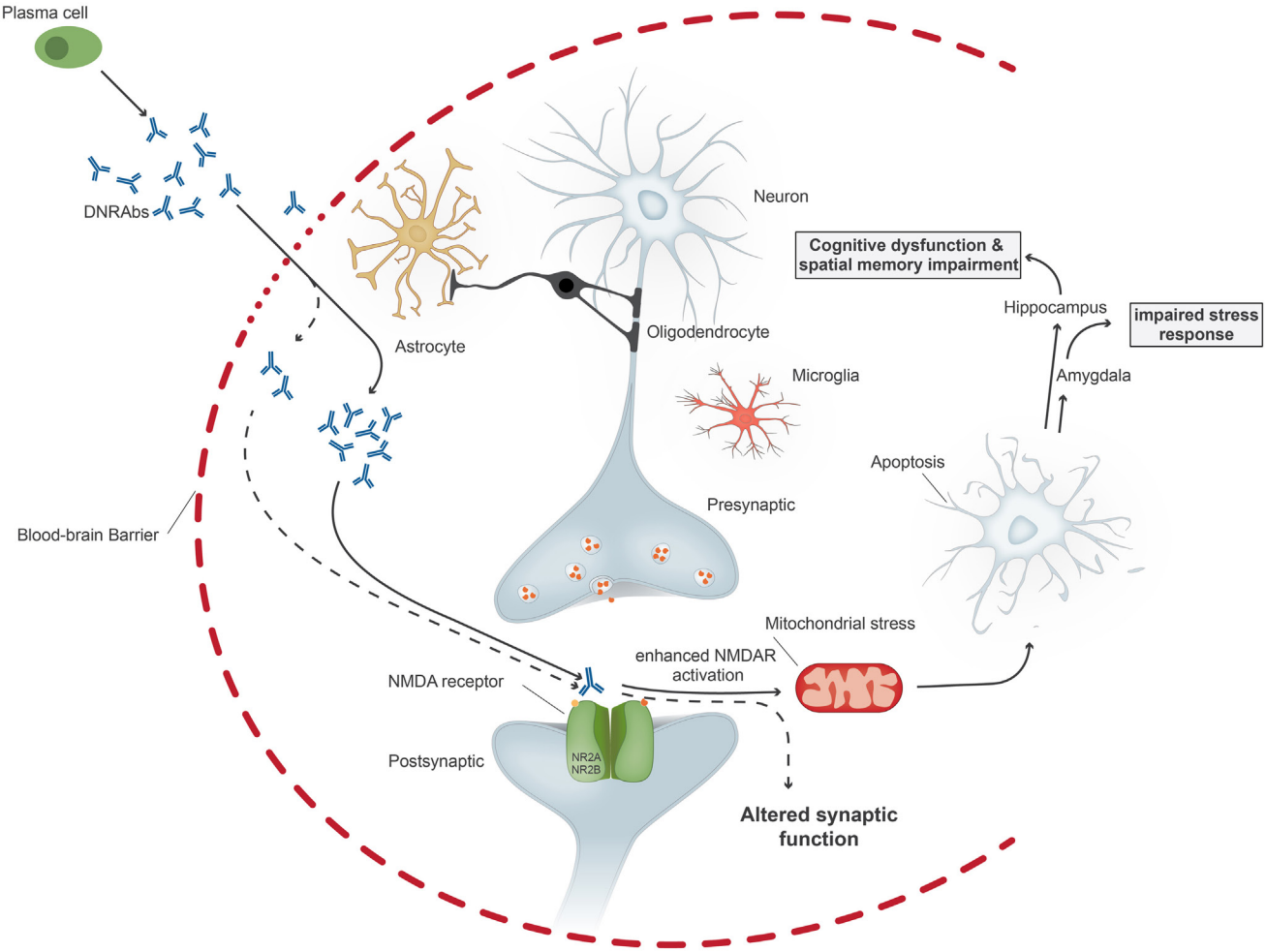

FIGURE 1 | Upon penetration of anti-DNA/anti-NMDA receptor antibodies (DNRAbs) into the central nervous system through the impaired blood–brain barrier, the antibodies trigger two different pathomechanisms dependent on the antibody concentration. At high concentrations (continuous line), the antibodies lead to mitochondrial stress and leads to neuronal apoptosis through receptors containing GluN2A. If the neuronal loss occurs in the hippocampus, the antibody binding results in spatial memory impairment and cognitive dysfunction. Binding of antibodies in the amygdala affects the stress response. At lower concentrations (dotted line), antibody binding results in altered synaptic function.

model in which pregnant mice expressed DNRAbs throughout gestation (30, 64). Maternal DNRAbs antibodies caused neuronal death in the fetal neocortex and resulted in cortical abnormalities and cognitive impairment in the adult male offspring. In contrast to the cognitive impairment observed in male mice, maternal DNRAbs resulted in increased death of female fetuses, thereby skewing the gender ratio of living offspring (30, 64). We showed that there was no difference in transplacental transfer of the pathogenic antibodies to male or female fetal brain. The gender-dependent effect may be explained by an increased expression of GluN2A in the fetal female brainstem during development compared to male littermates, or to gender-dependent differences in the vulnerability of fetal neurons to GluN2A signaling (64). Neutralization of pathogenic antibodies during pregnancies, perhaps by decoy antigen, may prevent neurodevelopmental impairment.

It should be noted that other antibodies present in SLE patients may affect fetal neurodevelopment. For example, antiphospholipid antibodies can lead to placental problems affecting fetal growth or fetal loss. Moreover, a study suggested that learning disabilities in children born to a mother with SLE were associated with high titers of maternal antiphospholipid antibody (65).

Astrocyte-Directed Antibodies in NMO

While most anti-brain antibodies target epitopes expressed on neuronal cells, anti-brain antibodies can also be directed to antigens expressed on non-neuronal cells, and thereby cause different brain pathology. In NMO, anti-brain antibodies bind to a protein expressed on astrocytes. NMO is a neurological autoimmune disease that is characterized by the presence of antibodies that bind to the water channel protein AQP4 (66), which is expressed on astrocytic endfeet that surround blood vessels. AQP4 is

particularly expressed at the BBB interface. Approximately 80% of NMO patients harbor AQP4 antibodies and the presence of AQP4 antibodies has important diagnostic and prognostic significance (67, 68). These antibodies are conformation dependent and can be detected with highest sensitivity using a cell-based assay with HEK cells expressing AQP4 on their cell surface (67, 69). The presence of AQP4 antibodies differentiates NMO from MS, which have overlapping clinical symptoms, particularly at disease onset. It is of high importance to differentiate MS from NMO since they benefit from different treatment choices (70). Several studies consistently showed that AQP4 antibodies are not present in MS patients or healthy controls and if found they predict development of NMO (68). Thus, AQP4-IgG serostatus has been included in the diagnostic criteria for the disease (38).

Neuromyelitis optica patients have lesions in areas of high AQP4 expression, such as the brain, optic nerve, and spinal cord (71). Histological findings show antibody deposition around blood vessels in the brain of patients (72). The disease primarily presents with astrocyte loss, inflammation with infiltration of granulocytes, macrophages and T cells, deposition of antibodies

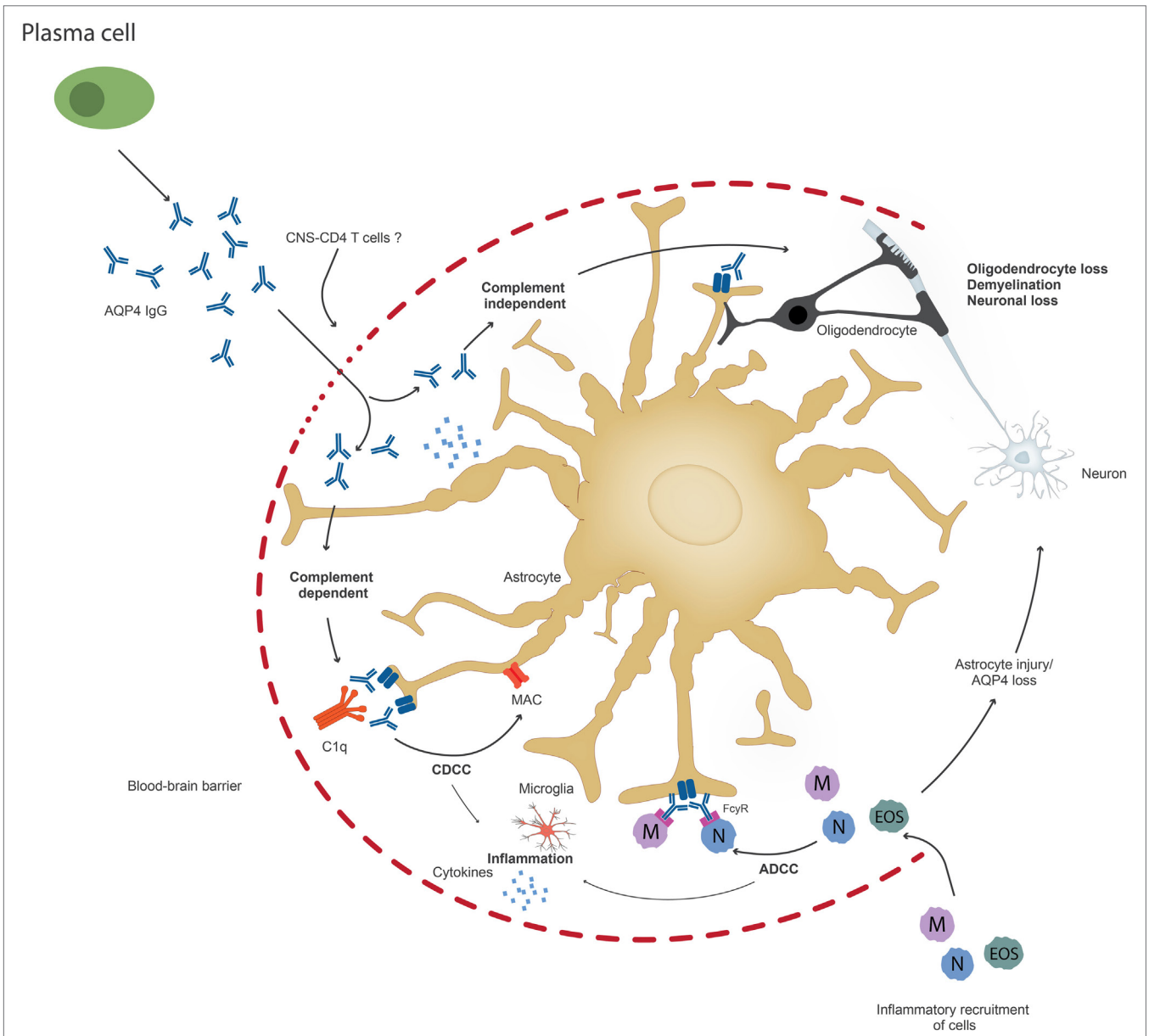

FIGURE 2 | Upon penetration of aquaporin-4 (AQP4) antibodies to the central nervous system, possibly through the help of pathogenic T cells, AQP4 antibodies can lead to astrocyte impairment either through complement-dependent cellular cytotoxicity (CDCC), antibody-dependent cytotoxicity (ADCC), or through downregulation of AQP4. Inflammation might occur prior to or secondary to antibody penetration. Ultimately, oligodendrocytes are affected resulting in demyelination and neuronal loss.

and complement around blood vessels and, in a later stage of the disease, demyelination, neuronal loss, and scar formation (72, 73). It remains to be investigated how AQP4-IgG that binds to astrocytes can damage oligodendrocytes and how the demyelination observed in NMO occurs. AQP4 antibodies are produced in the systemic circulation of patients and can be found at high serum titers in the CSF (74, 75). It has been suggested that AQP4 antibodies are produced through molecular mimicry to certain microbes (76), a hypothesis, which needs to be further investigated.

Several *in vitro* and *in vivo* models show a pathogenic effect of the AQP4 antibody either by itself, in association with pathogenic T cells, complement or different cytokines and chemokines (28, 77–81) It is possible that AQP4-IgG acts through multiple mechanisms, as suggested by pathological findings showing that, within the same patient, complement deposition is present in some active NMO lesions, while other lesions lack complement deposition (82). In current rodent models, either AQP4-IgG is injected directly into the brain or the BBB is breached prior to antibody injection, often by autoimmune encephalitis (EAE), administering activated autoreactive T cells directed to different CNS antigens (79, 83). In the human disease, we do not know how antibodies enter the brain. It has been suggested that circumventricular organs might be a possible route of entry, supported by findings of NMO lesions in these areas particularly at disease onset (84). Antibodies directed to glucose-regulated protein 78 were recently associated with BBB disruption in NMO and might facilitate penetration of AQP4-IgG antibodies into the CNS (27).

Once AQP4 antibodies penetrate the brain, they bind to astrocytes and trigger CDCC or ADCC (37) (**Figure 2**). It has been suggested that these two mechanisms result in astrocyte loss and inflammation and cause or increase BBB damage, which leads to further oligodendrocyte injury and demyelination, which finally results in neuronal loss (37). However, it is also possible that inflammation occurs prior to AQP4 IgG infiltration. It is possible that pathogenic T cells, maybe AQP4 specific or directed to other CNS antigens, are important not only to facilitate antibody production, but also for BBB disruption and may be required for astrocyte and neuronal damage. Current animal models do not closely resemble human patients with respect to the size and location of NMO lesions (13). This discrepancy could be caused by the choice of the target antigen of pathogenic T cells in animal models (85). It is also possible that NMO patients harbor not only AQP4 antibodies but also antibodies to neuronal antigens, which may or may not contribute to disease pathology. More studies are needed that address the role of other antibodies, microglial activation, and proinflammatory cytokine secretion, which could also be responsible for irreversible disease damage observed in NMO, possibly even when the AQP4-IgG antibodies are no longer present in the brain. There is evidence that AQP4-IgG also affects AQP4 function (86).

Cognitive dysfunction has only been recently assessed in NMO patients and needs further investigation (87–89). NMO patients have a different frequency and pattern of cognitive impairment compared to MS patients, suggesting different mechanisms of brain injury (90). Further animal models are needed to study if AQP4 antibodies contribute to cortical neuronal loss and if they can lead to cognitive impairment.

Current studies are trying to develop less invasive treatment options for NMO patients to bypass the highly immunosuppressive treatment (37). One possibility would be to block the AQP4 antibodies or their mode of action. Recently, Eculizumab, a monoclonal antibody that inhibits the classical complement pathway, has been shown to be an effective treatment in an open-label study, suggesting an important role for CDC in NMO (91). However, other effector pathways cannot be ruled out. Thus, it is very important to understand the direct effect of AQP4-IgG on tissue pathology, whether there are other pathogenic effector mechanisms and what processes may be initiated by antibody but continue even when antibody is no longer present. Targeting acute exposure of AQP4-IgG through inhibiting CDCC or/and ADCC might not be sufficient to prevent tissue destruction.

MATERNAL AQP4 ANTIBODIES IN NMO PREGNANCIES

Neuromyelitis optica patients have only recently been shown to have an increased frequency of miscarriages (92). Larger follow-up studies are needed to investigate the long-term effect of *in utero* exposure to AQP4-IgG on children of NMO patients, but there are case studies suggesting that maternal AQP4 IgG might result in birth defects (92, 93). Since astrocytes are expressed rather late in development, it is possible that AQP4 is expressed on astrocyte precursor cells during embryonic development.

CONCLUSION

Determining the mechanism of action and pathogenicity of several brain-reactive autoantibodies could facilitate more accurate and rapid diagnosis and enable novel treatment options. Here, we describe two examples of autoimmune diseases, which are mediated, at least in part, by autoantibodies and their pathology is well characterized. Both antibodies in those diseases are targeting extracellular antigens on brain cells, either neurons or astrocytes, but differ in their mechanism(s) of action, and hence their pathology.

Anti-DNA/anti-NMDA receptor antibodies in SLE are targeting neurons, resulting in neuronal cell death by enhancing NMDAR activation. Depending on the localization of BBB impairment, DNRAbs result in different neurocognitive or neurobehavioral phenotypes. There is no CNS inflammation following acute exposure to DNRAbs. The sustained, chronic state of neuronal damage secondary to neuronal death that persists after antibody exposure is no longer present in the CNS may reflect either neuron intrinsic effects secondary to antibody exposure or microglial activation.

Brain antibodies in NMO bind to the astrocyte water channel protein AQP4 and result in irreversible astrocyte damage due to CDCC or ADCC. There is increasing evidence that AQP4-IgG can also act by themselves and result in reversible internalization of the AQP4-IgG complex, which is coupled to the excitatory amino acid transporter (EAAT2) endocytosis (94). Different mechanisms might contribute to reversible and irreversible tissue damage of NMO patients [**Figure 2**, modified from Ref. (37)]. NMO is an example

of an antibody-mediated disease where brain pathology of patients shows an inflammatory infiltrate in the CNS, yet, the role of pathogenic T cells in the disease pathogenesis remains to be investigated.

In order to enable appropriate and less invasive treatment, we need to understand the acute and chronic effects of antibody exposure.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the review.

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REVIEW
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Sense and Immunity: Context-Dependent Neuro-Immune Interplay

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The sensory nervous and immune systems, historically considered autonomous, actually work in concert to promote host defense and tissue homeostasis. These systems interact with each other through a common language of cell surface G protein-coupled receptors and receptor tyrosine kinases as well as cytokines, growth factors, and neuropeptides. While this bidirectional communication is adaptive in many settings, helping protect from danger, it can also become maladaptive and contribute to disease pathophysiology. The fundamental logic of how, where, and when sensory neurons and immune cells contribute to either health or disease remains, however, unclear. Our lab and others' have begun to explore how this neuro-immune reciprocal dialog contributes to physiological and pathological immune responses and sensory disorders. The cumulative results collected so far indicate that there is an important role for nociceptors (noxious stimulus detecting sensory neurons) in driving immune responses, but that this is highly context dependent. To illustrate this concept, we present our findings in a model of airway inflammation, in which nociceptors seem to have major involvement in type 2 but not type 1 adaptive immunity.

Keywords: allergy and immunology, sensory neurons, asthma, inflammation, neuro-immunological signaling

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INFLAMMATION IS A NERVOUS THING

Inflammation was first characterized by Celsus as having four cardinal signs, *dolor* (pain), *calor* (heat), *rubor* (redness), and *tumor* (swelling) (1). Although we typically think of inflammation as an immune-mediated phenomenon, each of these characteristics is essentially due to neuronal activation. The local depolarization produced by the direct activation of membrane transducers in the peripheral terminals of nociceptors by noxious stimuli initiates action potentials which travel orthodromically from the periphery to the central nervous system to initiate reflexes (withdrawal, scratching, cough) and sensations (pain, itch) (2, 3). However, when the action potentials reach branch points of the sensory neurons they are also transmitted antidromically back to the peripheral terminals in a process known as the *axon reflex* (4, 5). The local depolarization and the action reflex are the means by which neurogenic inflammation is initiated. Calcium influx through voltage-gated calcium channels triggers the rapid and local release of neurotransmitters from activated peripheral terminals or those invaded by antidromic action potentials (4, 5). Neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P (SP), act on endothelial and smooth muscle cells to produce redness and heat (secondary to vasodilation) and neurogenic edema (secondary to plasma extravasation due to increased capillary permeability) (6, 7). This neurogenic component of the response to acute injury has long been recognized. However, the sensory neuronal involvement goes beyond contributing just to the vascular component of the inflammatory response to infection or injury. Complex reciprocal interactions between the sensory nervous and the immune systems have

recently been detected that drive both inflammatory pain hypersensitivity and immune cell recruitment and activation (2, 8).

An early indication that nociceptors play a major role in autoimmunity was the observation that denervation of a limb following a nerve injury prevented the subsequent development of arthritis in that limb (9). This clinical finding can be recapitulated in rodents, where eliminating sensory fiber innervation decreases inflammation in models of rheumatoid arthritis (10). Although denervation can protect from arthritis, blocking nerve activity can worsen experimental inflammation, as observed in a serum-transfer model of arthritis (11). These findings seem contrary, but may indicate that nociceptors play distinct roles during different phases of the immune response in arthritis; perhaps they are required for initiation of disease, but limit arthritic effector responses (mimicked in the serum-transfer model). During initiation of immune responses, the peptides produced by and released from the peripheral terminals of sensory neurons can direct the differentiation of lymphocytes (8, 12–18) and promote the recruitment of immune cells, and therefore may determine the localization, extent, duration, and type of inflammation taking place. In consequence, this neuro-immune interaction may result in a broad spectrum of different pathophysiological changes and disease states. Understanding the specifics of the interactions between sensory neurons and immune cells and defining the rules under which they operate will open new avenues for understanding immunity and for developing novel therapeutic strategies (2). In this perspective, we highlight context-dependent aspects of neuro-immune interactions involving sensory neurons.

PHYSIOLOGICAL BASIS FOR CONTEXT-DEPENDENT NEURO-IMMUNE COMMUNICATION

The primary goal of the immune system is to sense pathogens and respond accordingly for their effective removal, while limiting tissue damage and deleterious autoimmunity (19). To mount these responses, the immune system relies on an arsenal of immune cell subtypes specifically programed to eradicate a myriad of distinct pathogens. For example, different subpopulations of effector cells arise during activation of mature naïve $CD4^+$ T cells by innate cells responding to distinct environmental cues, yielding highly adaptable responses to the type of pathogen. Although they lack somatically re-arranged antigen receptors, different innate lymphoid cell (ILC) lineages also contribute to differential pathogen responses (20). Historically, immune responses have been classified into three distinct groups classified by the type of T helper cell induced: type 1 responses, which provide protection against intracellular microbes through, in part, the activation of phagocytes (21); type 2 responses, which defend against parasites such as helminths (also triggered by allergens, venoms, and toxins) (22); and type 3 responses that protect against extracellular pathogens (including yeasts/fungi) (23). Type 1 immunity is characterized by T-bet^+ IFNγ-producing ILC1, $CD8^+$ cytotoxic T cells, and $CD4^+$ Th1 cells which defend against intracellular virus and microbes through activation of $CD8^+$ cytotoxic T cells. Type 2 immunity is defined by GATA-3^+ ILC2, and Th2 cells that produce IL-4, IL-5, and IL-13, which induce mast cell, basophil, and eosinophil activation, as well as IgE antibody production (24). Type 3 immunity is mediated by ILC3s, and Th17 cells that produce IL-17, IL-22 (25), or both, which activate mononuclear phagocytes but also recruit neutrophils and induce epithelial antimicrobial responses.

For each of these different types of immunity, there are several stereotypical components. First, the insult must be "sensed." In the immune system, pattern recognition receptors (PRRs) have evolved to respond to a diverse array of stimuli (26). PRRs include TLRs, C-type lectin-like receptors, and NOD-like receptors, which are expressed on the surface and in intracellular compartments of a variety of cell types, including dendritic cells (DCs) and epithelial cells. Triggering PRRs activates DCs, which then stimulate antigen-specific T cells and tune the type of Th cell response required (i.e., type 1, 2, or 3). Indirect activation of DCs can also occur, for example, *via* epithelial cell-derived factors, such as thymic stromal lymphopoietin (TSLP), which perhaps conditions DCs to induce Th2 cell responses (27). Triggering type 2 immunity may also involve DC PRRs, enzymatic activity of allergens with intrinsic protease activity, recognition of tissue damage, or distinct metabolic changes caused by allergens or helminths (22).

Next, information must travel to peripheral immune tissues, such as spleen and lymph nodes, where it is transmitted to managerial cells—mostly T helper cells, which are instructed by innate immune cells to differentiate into effector populations. Typically, primed DCs, in addition to presenting antigen in the context of costimulatory molecules to naïve T cells (Th0), will also instruct naive T cells to differentiate into an appropriate effector type (Th1, Th2, Th17, and Treg) by releasing type-specific cytokines (22). For example, in a type 1 bacterial infection, DC make IL-12, IL-6, and IL-1β which drive Th1 cell differentiation (21); (b) while in a type 17 infection, TGF-β, IL-6, and IL-23 enhance ROR-γt and STAT3 activity and drive Th17 differentiation (23); (c) in type 2 inflammation, IL-4 from DCs, epithelial cells, basophils, $CD4^+$ Th2 cells, or ILC2 cells stimulates GATA-3 and STAT5, STAT6 to promote Th2 differentiation (22, 28, 29). Once polarized, the effector T cells travel back to the original location of the insult to direct appropriate host defense responses. They do so *via* the release of an "effector" sets of cytokines, such as IFN-γ by Th1 cells; IL-17, IL-21, and IL-22 by Th17 cells; and IL-4, IL-5, IL-9, and IL-13 by pro-inflammatory Th2 cells (24, 30). In disease states, these physiological responses are subverted to pathology. Each inflammatory disease is generally dominated by one particular type of immunity, which depends on the initial trigger and the individual's micro- and macroenvironment and genetic susceptibilities. For example, types 1 and 3 immunities mediate autoimmune diseases (rheumatoid arthritis and ulcerative colitis), whereas type 2 responses cause allergies (asthma and eczema).

WHERE DO NEURONS FIT INTO THIS MODEL?

The somatosensory nervous system is positioned anatomically to be able to directly modulate immunity in secondary and primary lymphoid tissues, skin, and mucosa, by interacting with

immune cells in locations where they are stationed to monitor for disturbances in barrier function and homeostasis (31–34). There is also crosstalk between the sensory neurons and the epithelial barrier (35), another important determinant of immune responses (36). In addition, sensory neurons express a wide array of ion channel receptors specialized for detecting danger signals. These danger signals can include excess heat or cold (TRPV1, TRPM8), chemical (TRPA1), and mechanical (Piezo2) insults (2, 5, 37). They can also sense protons (TRPV1) and the ATP released upon tissue damage and apoptosis (P2X3R). After neurons sense environmental cues, the acute sensory input travels to the CNS, prompting withdrawal and defensive reflexes (fever, cough, scratch, and vomiting) (2, 38–41).

Neuronal activation by the immune system has been fairly well studied. Nociceptors are activated by cytokines: IL-1β (42) or CCL3 (43) in the context of pain, IL-5 during allergic airway inflammation (8), IL-31 during lymphoma-associated itch (44), TSLP and IL-4 during atopic dermatitis (35), IL-33 after contact with poison ivy (45), and IL-23 during psoriasis (46). While TNF-α leads to the activation of nuclear factor κB (NF-κB) (47), MAP, and tyrosine kinases are typically downstream of IL-1R in sensory neurons (42). These kinases, including p38 (48), JAK1 (49, 50), and the transcription factor STAT3 (51), are just some of the downstream signaling molecules that lead to ion channel sensitization, a state of heightened activity. Much of nociceptor sensitization is related to a decrease in the activation threshold of TRPV1 or TRPA1 (52, 53), and of the sodium channels Nav1.7, Nav1.8, and Nav1.9 (54, 55), lowering nociceptor activation threshold results in pain hypersensitivity and increased itch, but also greater local release of neuropeptides that in turn activate immune cells to release more cytokines (2). Overall, inflammation leads to the simultaneous release of many pain sensitizing mediators. As a consequence, pharmacologically targeting only one of these agents may have limited effects. In addition, there is a negative feedback loop: IL-1β and IL-4 may, *via* the JAK–STAT axis, trigger increased expression of opioid receptors in neurons and, therefore, sub-acutely have anti-nociceptive and anti-inflammatory effects (56, 57).

PATHOGEN DEFENSE

We increasingly recognize that nociceptors play a much broader role in sensing their environment than originally thought; they can also directly detect bacteria and fungi, as well as may products of the immune response, including cytokines, chemokines, and immunoglobulins (2, 5, 37). Nociceptors express a variety of PRRs that directly recognize bacteria (58) and fungi (59, 60). *Staphylococcus aureus* is able to activate nociceptors through a twofold mechanism: (a) secreted pore forming toxins, for example, α-hemolysin, which permeabilizes the nociceptors' cell membrane allowing influx of extracellular cations and (b) *N*-formylated peptides that are membrane-bound peptides that activate their cognate receptors (formyl peptide receptors) on the surface of nociceptors (58). Following experimental ablation of these neurons, the pain associated with bacterial infection is, as expected, decreased, but surprisingly local immune infiltration is increased. These data suggest that bacteria may have evolved the capacity to activate and co-opt nociceptor function to dampen innate immune responses and facilitate their survival (58). However, the neuronal response may be distinct for different types of pathogens. In the context of *Escherichia coli* peritonitis infection, transection of the vagus nerve decreased ILC3 cell numbers, reduced pro-resolving mediator levels, and altered peritoneal macrophage numbers. Exogenous acetylcholine or pro-resolving mediators restored tissue resolution tone and host responses to *E. coli* infections (61). In intestinal organ cultures, capsaicin-sensitive enteric nervous system neurons seem to distinguish among different bacterial infections (62). For example, despite belonging to the same Clostridium bacterial subset, only a few nociceptors respond to *P. magnus*, while up to 60% of the capsaicin responsive nociceptors showed calcium flux when exposed to *C. Ramosum*. Interestingly, *P. magnus* downregulates genes encoding for SP, Secretogranin III, and galanin while it upregulates neurotensin and angiotensin. Intriguingly, *C. Ramosum* had the opposite effect (62). This seems to indicate that nociceptors may be able to change their activation depending on the type of immune response elicited by commensal vs pathogen and contribute in this way to either regulatory or type1/17 immunity.

Beyond their sensory role, neurons also promote host defense and do this in part by direct interaction with immune cells. The activation of sensory neurons results in the secretion of neuropeptides from their peripheral terminals that cause the recruitment, activation, and influx of immune cells (37, 63–66). CGRP and VIP can bias DCs to produce type Th1, Th2, or Th17-skewing cytokines and enhance DCs migration to the lymph nodes (67, 68). The same neuropeptide can have multiple different effects, in some cases biasing to Th1, Th2, or serving a regulatory role (13, 67). Along with SP, these neuropeptides can also act on Langerhans (63), Th2 (68, 69), and ILC2 cells (8, 16) to change their activation states in the skin during models of contact hypersensitivity or psoriasis, and in allergic airway disease models (8). For example, in Th2-immunity, SP released by itch transducing neurons is sensed by mast cells which degranulate and release secondary mediators such as histamine, causing swelling and further enhancing the neurogenic response (3, 18, 70). Skin is innervated both by sympathetic efferent and nociceptor afferent neurons, but the denervation produced by the ablation of nociceptors alone is sufficient to reduce contact sensitivity inflammatory responses (71). Brian Kim's group has studied the contribution of sensory neurons in AD-like skin inflammation induced by the topical irritant MC903 (calcipotriol) (50). They found that while the type 2 cytokines IL-4 and IL-13 directly activate (calcium flux) both mouse and human sensory neurons, they did not elicit acute itch. IL-4 sensitized subsets of neurons to respond to previously sub-threshold pruritogen levels (such as histamine), significantly increasing scratching. They hypothesized that, rather than acute itch, neuronal type 2 cytokine signaling promotes pathologic chronic itch and that interrupting these signals may represent an effective strategy to target itch. To test this, they generated the first sensory neuron specific cytokine receptor knockout mice (NaV1.8-Cre:IL-4R$^{fl/fl}$) and showed that IL-4$^{-/-}$ nociceptors mice are protected from AD-induced skin inflammation, displaying a distinct skin transcriptional profile characterized by reduced infiltration of Th2 and basophils

numbers. In a distinct skin inflammatory model, toxin-induced ablation of Nav1.8$^+$ nociceptors reverse imiquimod (*via* TLR7)-mediated psoriasis-like inflammation (46) while cutaneous denervation in psoriatic mice reduces the number of immune cells in lesions (72).

INFLAMMATORY REFLEX

While the sensory neuron axon reflex contributes to local tissue immune cell recruitment and activation, immune homeostasis is also under autonomic control, in the form of an "anti-inflammatory reflex." This systemic circuit starts when innate immune stimuli activate peripheral vagal afferents and terminates with efferent parasympathetic neurons inhibiting cytokine production by splenic macrophages, attenuating inflammation (32, 73). In the efferent arc, action potentials travel down the vagus in preganglionic motor fibers to the celiac ganglion to activate postganglionic adrenergic neurons that innervate the spleen. These neurons release norepinephrine, activating a special population of T cells that make acetylcholine (74). Acetylcholine binds to the α7nACHR expressed by splenic macrophages and inhibits their production of TNF-α (75). Harnessing the inflammatory reflex using bioelectronic devices (76), such as non-invasive vagal nerve stimulation, can help decrease the chronic inflammation found in rheumatoid arthritis patients (77) and mice with experimental inflammatory bowel disease (78). Similarly, non-invasive vagal nerve stimulation showed improved lung function in a small patient cohort by reducing exacerbations of bronchoconstriction (79).

AIRWAY IMMUNITY: A MODEL SYSTEM FOR DELINEATING NEURO-IMMUNE INTERACTIONS

While systemic inflammation detected by the vagus perhaps leads to an anti-inflammatory reflex, the local picture in the tissue is different. The vagus nerve innervates virtually all visceral organs, and nearly 20% of afferents neurons terminate within the airways (80). Although some lumbar neurons innervate the lung epithelium (81, 82), it is estimated that up to 95% of the innervation is of vagal origin (83). The vagal sensory neurons innervating the respiratory tract are situated in two distinct ganglia, the nodose and jugular ganglion which have distinct phenotypes, embryonic origins (neural crest vs placode), anatomical projections to the respiratory tract, and brain stem, and are likely to serve distinct functions. Most of the nodose ganglion afferent fiber express markers of nociceptors, including TRP channels (TRPA1, TRPV1, and TRPM8) (84), voltage-gated sodium channels (NaV1.7, NaV1.8, and NaV1.9), voltage-gated calcium channels (CaV2.2) and mechanosensitive channels (Piezo2) (85). While these nociceptors mostly serve a defensive role by detecting chemical, mechanical, or thermal threats and initiate essential, protective airway reflexes such as cough and bronchoconstriction (86), they may also directly respond to decreases in lung compliance leading to subconscious sighs or deep inspirations (87). Physiologically, airway nociceptors can evoke both cough and neurogenic inflammation, the latter being a consequence of the axon reflex discussed above (8, 88, 89).

There are several indications that nerves and immune cells talk to each other in the lung, and this communication may have special relevance in asthma. Asthma is a chronic inflammatory disease of the airway which is caused by a combination of environmental (90) and genetic factors (91). Asthmatic patients have a denser network of sensory fibers around airways (92, 93) and a reduced threshold for neuronal activation in response to airborne irritants (94) as well as increased neuropeptide levels in these neurons (95). Collectively these features indicate excessive activity of peptidergic sensory fibers during asthma (96). T cells clustering with nerve-contacting DCs proliferate only in the airways of mice with allergic inflammation but not in the airways of negative controls (97). Eosinophils also appear to cluster around airway nerves in patients with fatal asthma and in antigen-challenged animals (98), while eosinophil-derived basic proteins enhance activation of rat pulmonary sensory neurons (99). C-fiber denervation in rats decreases the numbers of DCs in the lung and pulmonary lymphatic immune cell influx (100) while lung nociceptor stimulation with capsaicin increases both neuropeptide release and immune cell infiltration (8, 100–104). Similarly, stimulation of sensory neurons with capsaicin in subjects with active allergic rhinitis produces a reproducible and dose-dependent leukocyte influx (105), while capsaicin desensitization reduces rhinitis allergen-challenge symptoms (106). SP, CGRP, VIP, and secretin all promote eosinophil chemotaxis *in vitro* (107). SP and neurokinin A are expressed by lung nociceptor afferents and released following a broad range of different stimuli, including allergens, ozone, or inflammatory mediators. Neurokinin 1 receptor blockade decreases mononuclear cells and neutrophils (108) and eosinophils in alveoli (109) while SP (110) or capsaicin (111) drive eosinophil influx, which suggests that sensory neuron-release of the neuropeptides may drive eosinophilia in both allergic asthma and hypersensitivity pneumonitis (112). Using single-cell RNA-seq, it was recently found that the Neuromedin U receptor 1 (NMUR1) was preferentially expressed by ILC2s after alarmin (IL-25) stimulation. Neuromedin U (NMU), the ligand of NMUR1, activated ILC2s *in vitro*, and *in vivo* co-administration of NMU with IL-25 strongly amplified allergic inflammation. Despite the limited lung innervation, NMU was found only expressed in lung afferent DRG neurons, not nodose vagal neurons, and the loss of NMU–NMUR1 signaling reduced ILC2 function, altered transcriptional programs following allergen-challenge *in vivo* and prevented the development of allergic airway inflammation (113).

Neurons also play a regulatory role in allergic airway inflammation; CD11c$^+$ airway mucosal DCs are in close contact with CGRP$^+$ nociceptors in both rodents and human subjects (63, 114). CGRP has also been shown to have a Th2-skewing preference (68, 115) CGRP-exposed DCs seemed to enhance Th2 type immunity (115, 116), increasing IL-4 production while decreasing Th1-associated cytokines IFN-γ and IL-2 (117–119). However, despite these reports, the majority of publications on the role of CGRP on DC in the lung suggest a predominant anti-inflammatory rather than pro-inflammatory effect. Pretreating DCs reduces the activation and proliferation of antigen-specific T cells and increases the

numbers of T regulatory cells (120). CGRP specifically inhibits the maturation of DCs *in vitro* while adoptive transfer of CGRP-pretreated DCs diminishes allergic airway inflammation *in vivo*, with reduced eosinophils and increased IL-10 in bronchoalveolar lavage fluid (BALF) (65).

As reviewed by Mazzone and Undem (80), the plastic nature of vagal sensory neurons in the lung typically leads to highly context-dependent responses to inflammatory cues. For example, (1) allergen-sensitization produces a vigorous increase in the number and amplitude of IL-5-medicated calcium responses in vagal nociceptors (8); (2) allergen-challenge triggers action potential firing in nodose ganglion nociceptors of allergen-sensitized guinea pig (80) and mice (121), but not in naïve animals; (3) allergen-challenge increased the excitability of A delta fibers to mechanical activation (122); (4) 24 h following an allergen-challenge, up to 25% of large neurofilament-positive nodose ganglion neurons innervating the respiratory tract start expressing SP and CGRP (93); (5) 24 h following BDNF exposure, nodose ganglion neurons innervating the trachea start express functional TRPV1 (123); and (6) allergen-challenge lowers the action potential firing threshold and increases the spiking rate of nucleus of the solitary tract (NTS) neurons, which is the brain-stem structure innervated by the vagus nerve that mediates many of its sensory functions (80). The context-dependent plasticity of nociceptors may be acute, as in the case of enhanced coughing following capsaicin or bradykinin delivery to the airways (124), or may be long-lasting due to changes in the CNS similar to the central sensitization following peripheral injury (125).

SENSORY NEURON ROLE IN Th1 vs Th2 IMMUNITY IN THE LUNG

Given the data indicating substantial plasticity and a diverse set of roles for sensory neurons in different inflammatory disorders in the lung, we decided to investigate the specific role of nociceptors in regulating immune cells in ovalbumin (OVA) and house dust mite models of allergic airway inflammation (8). In this study, we demonstrated an involvement of vagal sensory neurons in the murine Th2-skewed OVA model of allergic inflammation using aluminum hydroxide (AlOH) as adjuvant. This operated through a VIP–VPAC2 axis: nociceptors by releasing VIP drive VPAC2 expressing $CD4^+$ and ILC2 cells to release IL-5 which, in turn, activates sensory neurons in the lung to release more VIP (8). Similar results were obtained for the house dust mite model. This indicates that nociceptors amplify adaptive immune responses in the lung in response to allergen exposure in sensitized animals, at least in the setting of Th2 immune responses. We then found that temporary local pharmacological silencing of the nociceptors is sufficient to interrupt this pro-inflammatory signaling loop, a non-immune therapeutic strategy that has immunosuppresive action. This strategy aims to temporarily silence afferents in the adult lung using large pore ion channels as a drug entry port for charged sodium channel blockers to produce targeted action potential blockade only in activated nociceptors (126, 127). These charged molecules have no action extracellularly but block sodium channels when they get into the cell. Because of their cationic charge, they cannot permeate through the membrane but are small enough to enter cells through inflammation-activated TRPV1 and TRPA1 ion channels (126, 128).

Specifically, a single treatment with QX-314 (100 μM or 0.003%, nebulized for 20 min at 20 psi, day 18), a quaternary derivative of lidocaine, to OVA sensitized (day 0 and 7) and challenged (days 14, 15, 16, and 17) mice substantially reduces lung immune cell infiltration (day 21), with decreases in BALF numbers of leukocytes, eosinophils, macrophages, and lymphocytes (8). Building on these findings, we used this strategy to assess the influence of nociceptor ablation or silencing on the activity of primary immune drivers (ILC2 and DCs) of type 2 inflammation. Here, we generate new data showing that QX-314 abolishes the rise in lung ILC2 cells, ILC2-derived IL-5, as well as BALF inflammatory DC cells (**Figures 1A–C**). Thus, pharmacologic silencing of nociceptors results in decreased activation of ILC2s and Th2 effector cytokine production. The above protocol, sensitizing 8-week-old male BALB/c mice with OVA and aluminum hydroxide as adjuvant, produces a Th2-skewed inflammation (129). When mice are sensitized with a complete Freund adjuvant (CFA) and OVA, this produces non-eosinophilic Th1-skewed asthma (129). Remarkably in these mice, QX-314 treatment (100 μM or 0.003%, nebulized for 20 min at 20 psi, day 18) fails to impact the levels of $CD3^+$ cells (**Figure 1D**), macrophages (**Figure 1E**), and neutrophils (**Figure 1F**). These data show that the contribution of *vagal nociceptors on allergic inflammation is context-dependent* with respect to the type of initial immune priming, with substantial neuronal involvement in type 2 models of allergic airway inflammation but not in a type 1 model.

WHY WOULD NOCICEPTORS PARTICIPATE IN Th2 BUT NOT Th1 IMMUNITY?

Type 2 immune responses evolved to eliminate parasites and other organisms that cannot be taken care of by cell-mediated immunity. The best early defense against parasites are direct behavioral reactions elicited acutely by the parasite as a sensory-motor reflex arc; for example, the sensation of itch as a parasite invades the skin leads to a reflex action of scratching. Worms in the gut initiate peristalsis and, in the lung, parasites lead to cough and enhanced mucus production. We postulate that the neuronal response became associated with the type 2 immune response elicited by parasites in order to enable a coordinated defense response: release of histamine and IL-4 (which sensitize nociceptors), production of mucus (also due to joint neuro-immune effort), and of IgE antibodies. Thus, the linkage between sensation (airway irritancy) and behavior (cough) expanded to become a link between sensory neuron activation and immunity. Supporting this idea, allergens and other type 2 stimuli directly activate sensory neurons in tissues where parasites might be particularly active (lung mucosa, for example). An example: as discussed above, acetylcholine-producing neurons of the enteric nervous system are found in close proximity to ILC2 cells. In addition to their role in allergic inflammation, it was also recently reported that these neurons can directly sense worm products

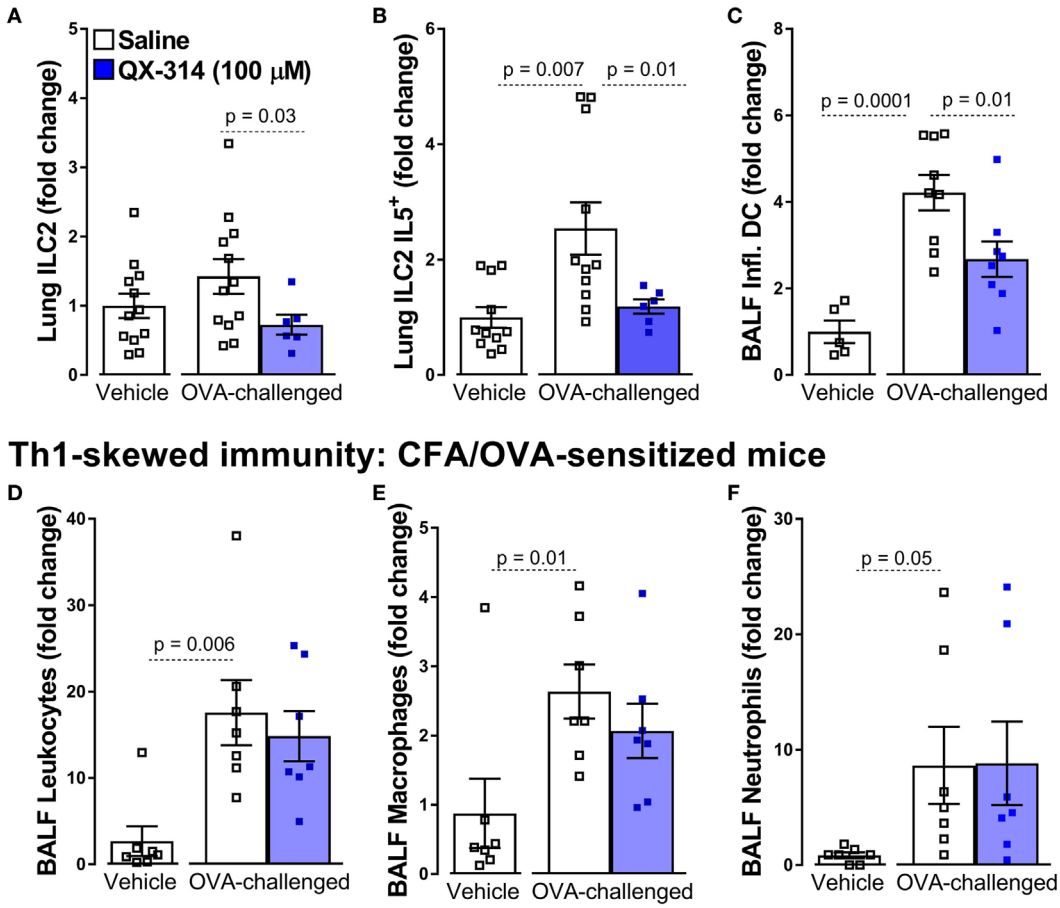

FIGURE 1 | Nociceptor activation promotes Th2 but not Th1 airway inflammation. In the aluminum hydroxide (AlOH)/ovalbumin (OVA) sensitized mice **(A–C)**, a standard model of airway Th2-driven inflammation, OVA-challenge do not significantly increase the numbers of ILC2 cells **(A)** in the lung but did enhance their production of IL-5 **(B)** as well as the numbers of inflammatory dendritic cells (DCs) in bronchoalveolar lavage fluid (BALF) **(C)**. Silencing lung sensory neurons with aerosolized QX-314 (0.003%, 20 min nebulization, 20 psi) decreased these Th2 immune cell responses. By contrast, silencing nociceptors in a Th1-driven lung inflammation model [complete Freund's adjuvant (CFA)]/OVA sensitized mice; **(D–F)**, had no impact on the OVA-challenge induced increases in BALF CD3$^+$ **(D)**, eosinophils **(E)**, and macrophages **(F)**. Mean ± SEM; *Two-tailed unpaired Welsh's t-test* (n = 5–12 animals/group; 1–2 cohorts).

(*N. brasiliensis* excretory/secretory products) and alarmins (IL-33), and, in turn, release neuromedin U to activate ILC2 cells. Thus, ILC2-autonomous ablation of Nmur1, the NMU receptor, impaired type 2 responses and control of worm infection. These data support a role for mucosal neurons to provide immediate tissue protection against worm products and alarmins and mount type 2 inflammatory responses (130). Future experiments will determine the extent of this linkage, and whether it holds true for other organs.

CONCLUDING REMARKS

As summarized in **Figure 2**, the influence of nociceptors on inflammation depends on the context (allergen sensitized or not) (80), the subtype of neuron activated, the subtype of immune cell that will respond to neuropeptide release, the location of the interaction (mucosal, epithelial, and endothelial), the timing of the interaction (acute vs chronic), and as our data suggests, the type of immune response (type 1 vs type 2). How, specifically, might nociceptors play different roles in different locations and in different types of immunity? One answer might lie in differences in expression of peptides and receptors. Nociceptors express receptors for 20 cytokines, 8 chemokines, and 6 immunoglobulins. They also express pattern recognition receptors, including two formyl peptide receptors, 11 toll-like receptors and 13 nucleotide-binding oligomerization domain-like receptors (131). Another example of the diverse functions of nociceptors is their expression of the immune checkpoint receptor PD-1 and their response to its cognate ligand (PD-L1) (132). PD-1 activation induces phosphorylation of the tyrosine phosphatase SHP-1, which inhibits sodium channels and decreases nociceptor sensitivity (132). Nociceptors also express in excess of 80 neuropeptides, each specific for receptors present at variable levels on various immune cells, creating the capacity to produce multiple different

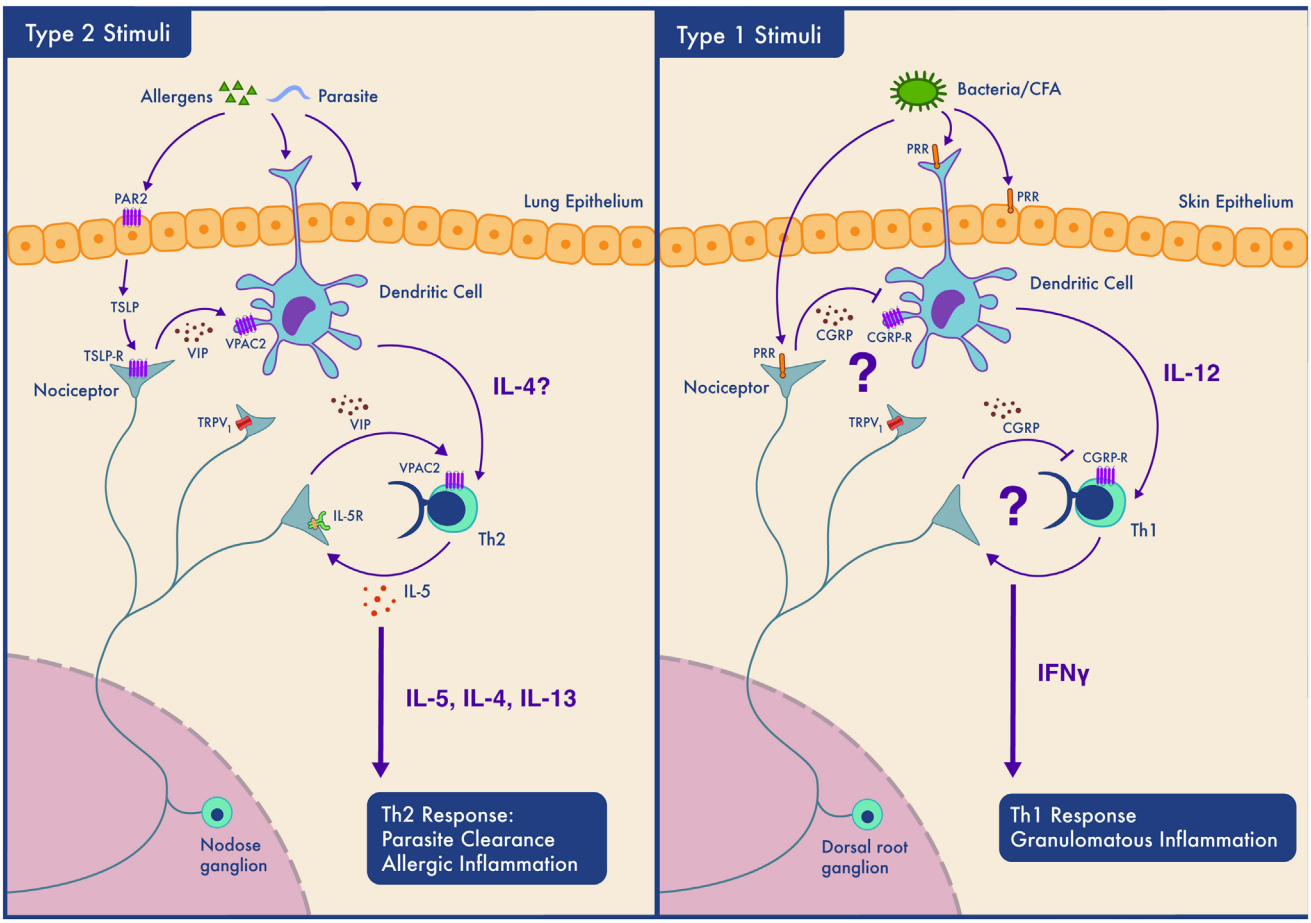

FIGURE 2 | Context-dependent neuro-immune interactions; nociceptors participate in Th2 but not Th1 type inflammation depending on the tissue type. Left panel. In type 2 inflammation, allergens or parasites are sensed by epithelial and dendritic cells (DCs). Epithelial cells may secrete mediators such as thymic stromal lymphopoietin (TSLP) which sensitize nociceptors. Nociceptors release neuropeptides including VIP which act on DCs and Th2 cells and contribute to their activation. Th2 cells secrete cytokines (IL-4 and IL-5) that both drive type 2 inflammation and act on nociceptors forming an inflammatory loop. While such inflammation perhaps aids in parasite clearance by promoting coughing and mucus secretion, if amplified and prolonged, this bidirectional communication contributes to the pathology of allergic inflammation. Right panel. The case is different in our model of Th1 type inflammation where the neuropeptides secreted by nociceptors do not activate immune cells and may even actively inhibit type 1 immunity. In this situation, nociceptors may sense pathogen-associated molecules and produce neuropeptides that limit DCs activation and downstream Th1 responses.

outcomes. The basal expression levels of both the neuropeptides and their cognate receptors vary in different inflammatory contexts and anatomical origins (lumbar vs vagal afferent) (131). Vagal nociceptors, for example, express higher level of Chrna5 (6.4-fold), TRPA1 (1.6-fold), VIP (16.2-fold), and IL-22R (9.2-fold) than somatic dorsal root ganglion neurons, while the latter express higher levels of KV7.5 (24.3-fold), TRPM8 (4.0-fold), CGRP (3.2-fold), and IL-31R (10.3-fold) (131).

Keeping in mind that immune cells themselves can release neurotransmitters such as dopamine by germinal center T_{FH} cells (133), we now need to examine in detail the broad repertoire by means of which sensory neurons and immune cells communicate with each other locally in damaged or infected tissue and explore where and how this contributes to disease. The development of new tools to monitor the *in vivo* activity of specific neuronal populations (by GCaMP6 and two-photon microscopy) and to stimulate (through optogenetics, DREADD), or block/ablate these neurons genetically (tetanus toxin, diphtheria toxin) or pharmacologically (QX-314), will help further dissect out the local sensory neuron–immune axis, especially when paired with single immune cell and neuron transcript profiling. Exploring the immune and nervous system contributions to inflammatory diseases will, we are confident, reveal novel therapeutic targets.

EXPERIMENTAL PROCEDURES

All procedures were approved by the Institutional Animal Care and Use Committees of Boston Children's Hospital. Mice were housed in standard environmental conditions (12 h light/dark cycle; 23°C; food and water *ad libitum*) at facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Allergic airway inflammation was studied in 8-week-old male BALB/c (stock number: 000651) mice using the classic OVA model (134) of asthma. On day 0 and 7, mice

were sensitized by a 200 µl i.p. injections of a solution containing 1 mg/ml OVA (Sigma-Aldrich) and 5 mg/ml aluminum hydroxide (AlOH; Sigma-Aldrich, Boston, MA, USA). On days 14–17 (10:00 a.m.) mice were exposed to 6% OVA aerosol for 20 min. We also investigated impact of sensory neuron silencing in a TH1-skewed model of allergic airway inflammation (129) following s.c. sensitization with OVA (1 mg/ml) in a 200 µl emulsion of sterile PBS and 50% CFA on day 0 and with 50% incomplete Freund adjuvant (IFA) on day 7.

Drugs

QX-314 (126) (Tocris) was diluted in sterile PBS to a 0.003% concentration (100 µmol) and mice were nebulized for 20 min at 20 psi on day 18 (8).

Bronchoalveolar Lavage Fluid

On day 21, a 20 G sterile catheter was inserted longitudinally into the trachea of deeply urethane-anesthetized mice (1.5 g/kg i.p.). Two milliliters of ice-cold PBS containing protease inhibitors were injected into the lung, harvested, stored on ice, centrifuged, cells isolated, and resuspended in sterile PBS (2, 58).

FACS

Single cells are isolated in FACS buffer (PBS, 2% FCS, EDTA), blocked (αCD16/CD32, 0.5 mg/ml, 10 min), and stained with specific monoclonal antibodies. Using a tiered gating strategy, cells are identified using light scatter parameters (FSC by SSC) and doublets are excluded. Cell populations are defined as follows: alveolar macrophages (sygF$^+$CD11b$^-$CD11c$^+$CD64$^+$), DCs (CD11c$^+$CD103$^+$CD24$^+$FcεR1$^+$), neutrophils (CD11b$^+$Ly6g$^+$), leukocytes (CD45$^+$CD3$^+$), and ILC2 (Lin$^-$Thy1$^+$ST2$^+$CD25^{Hi}) (2, 58, 135, 136).

Cell Count

Total BAL cell counts were performed using a standard hemocytometer, with absolute cell numbers calculated as total BAL cell number multiplied by the percentage of cell subpopulation as determined by FACS (134). Data are presented as fold change in comparison to control mice.

Intracellular Cytokine Staining

Cells were stimulated with PMA/Ionomycin in the presence of GolgiPlug (BD Biosciences) for 4 h and then fixed and stained using the BD Cytofix/Cytoperm kit following manufacturer's instructions (BD Biosciences) (8).

STATISTICS

Data expressed as mean ± SEM from 5 to 12 mice. Statistical significance determined by two-tail unpaired Welsh's *t*-test. *p*-values less than 0.05 were considered significant. Numbers of animals are indicated on the figure.

AUTHOR CONTRIBUTIONS

ST and SF designed, analyzed, and performed experiment. ST, CS, SF, and CW wrote the manuscript.

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Immunization Elicits Antigen-Specific Antibody Sequestration in Dorsal Root Ganglia Sensory Neurons

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The immune and nervous systems are two major organ systems responsible for host defense and memory. Both systems achieve memory and learning that can be retained, retrieved, and utilized for decades. Here, we report the surprising discovery that peripheral sensory neurons of the dorsal root ganglia (DRGs) of immunized mice contain antigen-specific antibodies. Using a combination of rigorous molecular genetic analyses, transgenic mice, and adoptive transfer experiments, we demonstrate that DRGs do not synthesize these antigen-specific antibodies, but rather sequester primarily IgG_1 subtype antibodies. As revealed by RNA-seq and targeted quantitative PCR (qPCR), dorsal root ganglion (DRG) sensory neurons harvested from either naïve or immunized mice lack enzymes (i.e., RAG1, RAG2, AID, or UNG) required for generating antibody diversity and, therefore, cannot make antibodies. Additionally, transgenic mice that express a reporter fluorescent protein under the control of Igγ1 constant region fail to express *Ighg1* transcripts in DRG sensory neurons. Furthermore, neural sequestration of antibodies occurs in mice rendered deficient in neuronal *Rag2,* but antibody sequestration is not observed in DRG sensory neurons isolated from mice that lack mature B cells [e.g., *Rag1* knock out (KO) or μMT mice]. Finally, adoptive transfer of *Rag1*-deficient bone marrow (BM) into wild-type (WT) mice or WT BM into *Rag1* KO mice revealed that antibody sequestration was observed in DRG sensory neurons of chimeric mice with WT BM but not with *Rag1*-deficient BM. Together, these results indicate that DRG sensory neurons sequester and retain antigen-specific antibodies released by antibody-secreting plasma cells. Coupling this work with previous studies implicating DRG sensory neurons in regulating antigen trafficking during immunization raises the interesting possibility that the nervous system collaborates with the immune system to regulate antigen-mediated responses.

Keywords: DRG, sensory neurons, antibodies, neural circuits, inflammation

INTRODUCTION

The mammalian immune system has acquired capacity to recall prior exposure to a vast number of potential antigens during the lifetime of the host. The development of this long-lasting immunity begins in a lymph node as an acute encounter between a naïve T cell and an antigen presenting cell. T and B lymphocytes recirculate between the lymphatic fluid, lymph node, and blood, entering the lymph node through high endothelial venules. Once inside the node, lymphocytes migrate through the T-cell zone and B-cell follicles until encountering an antigen presented by CD169+ macrophages or other antigen-presenting dendritic cells (1). The temporal and spatial control of antigen transport into the lymph node is dependent upon both the flow of isolated antigen *via* afferent lymphatic channels, and the trafficking into lymph nodes of intracellular antigen in antigen-presenting cells (APCs). Significant changes in lymph node architecture, inhibition of lymphocyte egress, and expansion of the lymph node stroma occur following antigen-mediated activation of intranodal immune responses.

We recently discovered that neural signals provide an essential mechanism in lymph nodes to retain antigen and lymphocytes (2). Lymph nodes are innervated by both sensory (afferent) and motor (efferent) neurons, with abundant innervation of the APCs in the T-cell zone, subsinoidal layer, and cortical extrafollicular zones (3, 4). The neuronal density within the lymph nodes is dynamic, expanding significantly following antigenic stimulation of an early lymphocyte response (5, 6). In naïve mice exposed to the antigen keyhole limpet hemocyanin (KLH) in the hind paw for the first time, we observed that KLH flowed rapidly from the popliteal lymph node (adjacent to the injection site) to the sciatic lymph node by traveling up the lymphatic chain. Surprisingly, prior exposure to KLH significantly impaired the flow of KLH from the popliteal to the sciatic lymph node during subsequent antigen exposure. This restriction of antigen flow from distal to proximal lymph nodes was antigen-specific and required functioning sensory neural signals, because inhibiting neural signals to the lymph node region restored antigen flow in immunized animals (2). The neural mechanism that restricted antigen flow is mediated by a subset of $Na_V1.8$+ sensory neurons, which include nociceptors, because antigen failed to accumulate in the distal lymph node of animals rendered deficient in $Na_V1.8$+ neurons (2). Early work had previously established that immune complexes can interact *via* Fc receptors expressed on sensory neurons to induce intracellular signaling mechanisms that require transient receptor potential canonical 3 (TRPC3) channel and the Syk-PLC-IP3 pathway (7, 8) that mediates the release of calcitonin gene-related peptide (CGRP) and substance P (9). Immunohistochemistry using labeled antigen revealed colocalization of labeled antigen and FcγRI receptors in neurons at the site of antigen injection in the paw (2). Thus together, these studies implicate sensory neurons in regulating antigen trafficking during immunization through a pathway that requires $Na_V1.8$ and FcγR.

As we continued to explore the role of sensory neurons in immunization, we were very surprised to observe that sensory neurons obtained from the dorsal root ganglia (DRGs) of immunized animals contained abundant levels of antigen-specific antibodies. While others have reported the localization of antibodies to neurons in brain, to our knowledge, the localization of antibodies to sensory neurons in peripheral tissues has not been previously described. *Rag2* expression has been identified in brain neurons, but convincing evidence that neurons can synthesize antigen-specific antibodies is lacking. Accordingly, here we used molecular genetic analyses, and transgenic and chimeric mice to show for the first time that dorsal root ganglion (DRG) sensory neurons in immunized animals accumulate lymphocyte-derived, antigen-specific IgG_1.

MATERIALS AND METHODS

Animals

Six- to eight-week old male C57BL/6J (CD45.2), congenic B6.SJL-Ptprc^a*Pepc*^{b}/BoyJ (also known as CD45.1;B6 Cd45.1, Pep Boy; or Ly5.1), B6.129S7-*Rag1*^{tm1Mom}/J [also known as *Rag1* knock out (KO)]; B6.129P2(Cg)-*Ighg1*$^{tm1(cre)Cgn}$/J (Cγ1-cre), B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J (Ai14(RCL-tdT)-D), C57BL/6-*Rag2*^{tm1Cgn}/J (Rag2 flox), and B6.Cg-Tg(Syn1-cre)671Jxm/J and B6.129S2-*Ighm*^{tm1Cgn}/J (μMT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Syn1-cre mice were crossed to *Rag2*$^{flox/flox}$ mice to generate Syn-cre/Rag2$^{flox/+}$. Syn1-cre/Rag2$^{flox/+}$ mice were backcrossed to Rag2$^{flox/flox}$ mice to generate Syn1-cre/Rag2$^{flox/flox}$ (Rag2^{KO}) mice. To generate Ighg1-tdTomato mice, B6.129P2(Cg)-*Ighg1*$^{tm1(cre)Cgn}$/J mice were bred with tdTomato reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J). Mice were housed in the animal facility of the Feinstein Institute for Medical Research under standard temperature and 12 h light and dark cycles. All mice were allowed to acclimate for at least 7 days before experimentation. All protocols used in this study involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research, and all experimentation was in accordance with the National Institutes of Health guidelines for animal care (Guide for the Care and Use of Laboratory Animals, National Research Council 2011).

Generation of Chimeric Mice

The recipient mice were sub-lethally irradiated with two split doses (WT: 5 Gy; *Rag1* KO: 4 Gy) delivered 14 h apart, and their immune systems were rescued *via* BM transplantations from *Rag1* KO (CD45.2) or WT (CD45.1) mice, respectively. Antibiotic gel diet (MediGel® TMS) was given to animals before and after irradiation. Donor mice were euthanized by CO_2 asphyxiation/cervical dislocation, and the femur and tibia were harvested. BM cells were collected by gently flushing the femurs and tibia and suspended at a concentration of 2×10^6 cells in 200 μL of sterile Hank's balanced salt solution (HBSS). Cells were administered intravenously (retro-orbital injection) to recipient mice 4–5 h after the second dose of irradiation. Reconstitution of the BM in recipient mice was confirmed after 2–4 weeks by assessing numbers of CD45.1 or CD45.2-positive cells in the peripheral blood by flow cytometry (reconstitution was >98%).

Immunization Protocols

For all immunohistochemical staining of DRGs, RNA-seq, and ELISpot assays, mice were injected intraperitoneally with either 50% alum (Imject™ Alum; Thermo Fisher Scientific, Waltham, MA, USA) or 50% alum + 100 μg KLH (Calbiochem, San Diego, CA, USA) or 50% alum + 100 μg ovalbumin (OVA; Invivogen, San Diego, CA, USA) in 200 μl of 0.9% saline on day 0 and day 14. DRGs were harvested 14 days later (see below). For *in vivo* analysis of gene expression by quantitative PCR (qPCR), mice were injected with either 50% alum or 50% alum + 100 μg KLH in 200 μl of 0.9% saline on days 0, 14, and 28, and DRGs were collected 24 h post each injection.

Collection of Whole DRGs and Isolation of Sensory Neuron Preparations

Dorsal root ganglia from thoracic and lumbar regions were harvested from mice immediately following euthanasia by CO_2 asphyxiation. DRGs from the thoracic and lumbar regions were chosen because they have been shown to innervate the peritoneal cavity (10). DRGs were gently collected from the cavities along the lateral vertebral column as described previously (11) and enriched for neuronal populations, as described by Ref. (12). For the isolation of sensory neurons, DRGs were digested with 1 μg/ml collagenase/dispase (Roche Life Science, Germany) in neurobasal medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 37°C on a rotator-shaker. Following digestion, DRGs were washed with HBSS (Gibco), triturated using fire-polished glass Pasteur pipettes (Fisher Scientific, Waltham, MA), and filtered through a 70-μm strainer. The cell suspension was layered onto 15% BSA in HBSS and centrifuged at ×129*g* for 20 min without brake. The cell pellet was re-suspended in neurobasal medium. B lymphocytes were depleted from cell preparations using Dynabeads™ mouse pan B purification kit (Invitrogen, USA), and cell purity was assessed by flow cytometry. The resulting sensory neuron preparation was resuspended in complete neurobasal medium [neurobasal™ medium supplemented with penicillin-streptomycin (Gibco), GlutaMax™ (Gibco), B-27® serum-free supplement (Gibco), and 50 ng/ml NGF (Sigma-Aldrich)].

Immunohistochemical Staining

Whole mount DRGs were harvested from mice immediately after euthanasia by CO_2 asphyxiation as described above and fixed with 4% PFA for 2 h. Fixed specimens were incubated overnight in 30% sucrose/PBS, washed, embedded, and frozen in optimal cutting temperature compound (Tissue-TEK OCT, Electron Microscopy Sciences, VWR, Radnor, PA, USA). 14 μm thick frozen sections were mounted on the Superfrost Plus glass slides (Thermo Fisher Scientific). The tissue sections were blocked and permeabilized with blocking solution containing rabbit serum and 0.1% Triton X-100. For immunostaining, blocked/permeabilized sections were stained with AlexaFluor 488-rabbit $F(ab')_2$ anti-mouse IgG (H + L) (1:1,000; Abcam, San Francisco, CA, USA), AlexaFluor 647-rabbit anti-NeuN (1:50; Abcam), and DAPI. Sections were mounted in ProLong anti-fade mounting medium (Thermo Fisher Scientific) and imaged using an LSM880 laser scanning confocal microscope (Carl Zeiss) using X40 Zeiss plan-apochromatic oil objective with Z stacks. The numbers of NeuN-positive and NeuN-positive IgG-positive neurons were quantified in five DRG sections pooled from three animals using ImageJ. The percentages of IgG-positive neurons were quantified by counting the number of neurons double positive for IgG and NeuN divided by the total NeuN-positive neurons in each DRG section and then multiplied by 100.

Primary Cultures of Isolated DRG Sensory Neuron Preparations

Dorsal root ganglion neuron preparations were cultured in complete neurobasal medium [neurobasal™ medium supplemented with penicillin-streptomycin (Gibco), GlutaMax™ (Gibco), B-27® serum-free supplement (Gibco), 50 ng/ml NGF (Sigma-Aldrich), 10% fetal bovine serum, and 1× β-mercaptoethanol] for DRG neuron activation assays (see below).

ELISpot Assay

Dorsal root ganglion sensory neurons (isolated and prepared as described above) were cultured on anti-IgG or OVA- or KLH-coated ELISPOT plates and developed using the standardized ELISpot kit (Mabtech Inc., Cincinnati, OH, USA). Briefly, 96 well flat bottom multi-screen filter plates (Millipore, Billerica, MA, USA) were coated with 100 μL per well of KLH (50 μg/ml) or OVA (50 μg/ml) or anti-IgG antibody (15 μg/ml). The plates were incubated overnight at 4°C, washed with PBS, and blocked using complete neurobasal medium. DRG sensory neurons (isolated and prepared as described above from five mice per group for eight wells) suspended in complete neurobasal medium were cultured in the plates overnight at 37°C with 5% CO_2. ELISpots were developed as per the manufacturer's protocol and scanned and analyzed at Cellular Technology Ltd. (Shaker Heights, OH, USA).

FluoroSpot Assay

Dorsal root ganglion sensory neurons (isolated and prepared as described above) were cultured in anti-mouse IgG-coated plates and incubated overnight at 37°C with 5% CO_2. FluoroSpots were developed using the fluorescent tagged mouse IgG_1, IgG_{2a}, and IgG_3 detection kit as per the manufacturer's protocol (Cellular Technology Ltd.) and scanned and analyzed at Cellular Technology Ltd.

In Vitro B Cell and DRG Neuron Activation Studies

Splenic B cells were purified from 7- to 10-week-old naïve C57BL/6 mice using the EasySep B Cell Isolation Kit (STEMCELL Technologies, Cambridge, MA, USA). B cell purity (CD19-positive cells) was confirmed (>99%) using flow cytometry. Isolated B cells were cultured in complete DMEM containing penicillin, streptomycin, glutamine, β-mercaptoethanol (1× each) and 10% fetal bovine serum at 3×10^6 cells/ml per well (24 well plate) in duplicate. DRG neurons were isolated from naïve C57BL/6 mice and depleted of B cells, as described above. DRG neurons from five naïve mice were cultured in 0.25 ml per well (48 well plate) in duplicate per condition in complete neurobasal

media (described above) containing 10% fetal bovine serum and 1× β-mercaptoethanol. B cells and DRG neurons were stimulated with (1) vehicle (untreated cells); (2) LPS (25 μg/ml) + IL-4 (10 ng/ml); and (3) anti-CD40 (10 μg/ml) + IL-4 (10 ng/ml). B cells were harvested 24, 48, and 72 h post stimulation, and DRG neurons were harvested 48, 72, 96, and 120 h post stimulation, and markers of antibody production and the generation of plasmablasts/plasma cells were assessed by qPCR (e.g., *Rag2, Aicda,* and *Prdm-1*, as described below; see **Table 1** for primers).

RNA Isolation

Naïve mouse spleens (positive control) and DRG neuron preparations isolated from naïve, alum-injected, and alum + KLH-immunized mice ($n = 10$ mice per condition, pooled) were lysed with Qiazol lysis reagent (Qiagen, Germantown, MD, USA) and stored at −80°C until RNA preparation (DRG neurons from 10 mice yielded approximately 50 μg RNA). Spleen and DRG RNA were isolated using the RNeasy Universal Kit (Qiagen). For the *in vitro* activation assays (described above), RNA was isolated from DRG neurons using the RNeasy Universal Kit (Qiagen) and from B cells using the RNeasy Mini Kit (Qiagen). For RNA-seq studies, RNA quality was analyzed using the Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA); RNA integrity numbers (or RIN values) were approximately 8.0. All RNA samples had OD260:OD280 ratios >1.8.

RNA-Seq and Analysis

The sequencing mRNA libraries (for paired-end reads) were prepared using RNA isolated from DRG neurons purified from either alum-injected or alum + KLH-immunized C57BL/6J mice ($n = 10$ mice per group, pooled) using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. As an exploratory step for characterizing the DRG neuron preparations, deep RNA sequencing was performed on the Illumina NextSeq 500 platform. The read depth was at least 60 M reads per sample using 75 base pair paired-end reads for a total output of ~18 Gb. The raw image files from the NextSeq sequencer were de-multiplexed and converted to fastq files using the bcl2fastq BaseSpace App (Illumina). RNA-seq analysis was performed on the existing annotated reference transcriptome (mouse) – no alternate transcript discovery was performed. Adapter sequences and low-quality base reads were trimmed from fastq files using cutadapt. The fastq files were aligned to the iGenome mm10 reference sequence from Illumina, and gene expression was quantified using kallisto.[1] Data are reported as transcripts per million (TPM), which are considered more comparable between samples of different origins and composition, and the most frequently reported RNA-seq gene expression values (13). An expression value >3.0 TPM (transcripts per million) was used as a threshold for detectable expression. The RNA-seq data discussed here have been deposited in the NCBI Gene Expression Omnibus (GEO) (14) and are accessible through GEO Series accession number GSE108428.[2]

TABLE 1 | Quantitative PCR (qPCR) primers and probes.

Gene name	Forward sequence	Reverse sequence	Probe
Rag1	aggcctgtggagcaaggta	gctcagggtagacggcaag	46
Rag2	tgaataaagatgtcaacagccaat	ggtaccctcaatccccactt	29
Aicda	tcctgctcactggacttcg	gcgtaggaacaacaattccac	71
Ungv1v2	ccatggggatttgtcagg	acagtgaggacggcgttg	101
Cd19	aaggtcattgcaaggtcagc	ctgggactatccatccacca	21
Cd138	gagggctctggagaacaaga	tgtggctccttcgtccac	5
Prdm1	cgctatgactttggtgcttg	accctcacctctgcactga	108
Hprt1	tcctcctcagaccgctttt	cctggttcatcatcgctaatc	95

Forward and reverse primers and probes for indicated mouse genes (Roche Universal ProbeLibrary); v1v2 = variant 1 variant 2.

RNA-Seq Deconvolution Analysis

We utilized DeconRNASeq software (15) to perform RNA-seq deconvolution analysis to infer cell type compositions of the neuronal preparations isolated from alum-injected and alum + KLH-immunized mice. DeconRNASeq, a freely available software package written in R, was downloaded from http://bioconductor.org/packages. DeconRNASeq uses a globally optimized non-negative decomposition algorithm for estimating the relative proportions of distinctive cell types in a mixture sample. High-quality pure cell-type signatures were derived from RNA sequencing data on single cells from the mouse lumbar DRGs (16). The data are available from the GEO (GSE59739), and the normalized data expressed as reads per million (RPM) were downloaded from http://linnarssonlab.org/drg/. This dataset contains single cell RNA-seq measures of 799 cells as well as clustering assignments of 731 of these cells by principal component analysis. The five principal cell classes included 109 non-neuronal (NoN) cells, 81 peptidergic nociceptor (PEP) cells, 169 non-pep (NP) cells, 139 neurofilament containing (NF) cells, and 233 tyrosine hydroxylases containing (TH) cells. We averaged the RNA-seq measures for each of the five cell classes. We excluded genes in which the mean RPKM (reads per kilobase of TPM mapped reads) value for the cells types was below a 0.1 cutoff value. There were 11,776 genes that passed this constraint and were used in the deconvolution analysis. Using the deconvolution analysis, we analyzed relative proportions of distinctive cell types in our groups. Based on pure cell-type signatures, approximately 73% of our DRG neuron preparations were neuronal (Figure S1 in Supplementary Material). Of the cells with a neuronal origin, three different neuronal subtypes were identified: peptidergic nociceptor (PEP) cells, non-PEP (NP) cells, and NF (NF) cells.

Two Step Real-Time qPCR

Two step qPCR analysis was carried out to analyze the relative expression of target genes in B cell-depleted DRG neuron preparations isolated from naïve, alum-injected and alum + KLH-immunized mice, spleens and whole DRG preparations (without B cell depletion) isolated from naïve mice, as well as *in vitro* stimulated B cells and DRG neuron preparations (described above). First, cDNA synthesis was performed using ~1.5 μg of RNA using the ABI High-Capacity cDNA Reverse Transcription Kit (Thermo

[1] http://www.nature.com/nbt/journal/v34/n5/full/nbt.3519.html (Accessed: December 29, 2017).

[2] https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108428 (Accessed: December 29, 2017).

Fisher Scientific). Second, qPCR reactions were performed (up to 35 cycles) in triplicate using the specific mouse primer pairs and probes (**Table 1**) (Roche Universal ProbeLibrary), ~25 ng cDNA per well in a 384-well plate using the Eurogentec qPCR MasterMix (AnaSpec, Fremont, CA, USA) and the Roche 480 Light Cycler (Roche Diagnostics). All qPCR samples were run in triplicate. Mouse *Hprt1* was used as the housekeeping gene for normalization. Changes in gene expression, normalized for *Hprt1* expression, were calculated as relative fold-changes using the comparative Ct (ΔΔCt) method (17).

Statistical Analysis

GraphPad Prism 6.0 software was used for all statistical analysis. Values are presented as mean ± SD. Kruskal–Wallis ANOVA, followed by appropriate *post hoc* tests for multiple comparisons, and Mann-Whitney non-parametric test (for two groups) were performed to determine statistical significance. *P* values equal to or below 0.05 were considered significant.

RESULTS

Detection of IgG in Sensory Neurons of Mouse DRG

To study the accumulation of IgG in DRG sensory neurons, we performed immunohistochemical analysis of whole mount DRGs collected from naïve (untreated), alum-injected, or alum + KLH-immunized mice using a rabbit $F(ab')_2$ anti-mouse IgG (H+L) antibody. The $F(ab')_2$ fragment against IgG has been used for immunohistochemical staining because it is small and can penetrate tissues/cells, and does not bind to the Fc receptors expressed on the target cells (18, 19). Mice were subjected to either sham immunization, intraperitoneal injection with adjuvant (alum) or immunization with alum + KLH. Animals received an intraperitoneal booster dose on day 14, and whole mount lumbar and thoracic DRGs were isolated from naïve and immunized mice after 4 weeks of immunization. Neuronal populations from DRG sections were stained with labeled anti-NeuN antibodies that recognize the neuronal nuclear protein NeuN. While only sparse IgG-positive staining was observed within the NeuN-positive DRG sensory neurons from the naïve and alum-injected animals, a significant increase in the number of double IgG-positive NeuN-positive neurons was observed in DRGs collected from alum + KLH-immunized mice (**Figures 1A–C**). Quantitative analysis revealed that IgG-positive staining of the NeuN+ DRG neurons was similar in naïve (3.5% ± 1.8%,) and alum-injected mice (5.9% ± 4.1%) (**Figure 1D**, a total of 824 and 1,343 NeuN+ neurons were analyzed from naive and alum-injected mice, respectively). By contrast, a significantly higher percentage of NeuN+ DRG neurons stained for IgG following alum + KLH immunization (23.8% ± 9.1%) (**Figure 1D**, $P < 0.05$, a total of 814 NeuN+ neurons were analyzed). NeuN is most abundant in

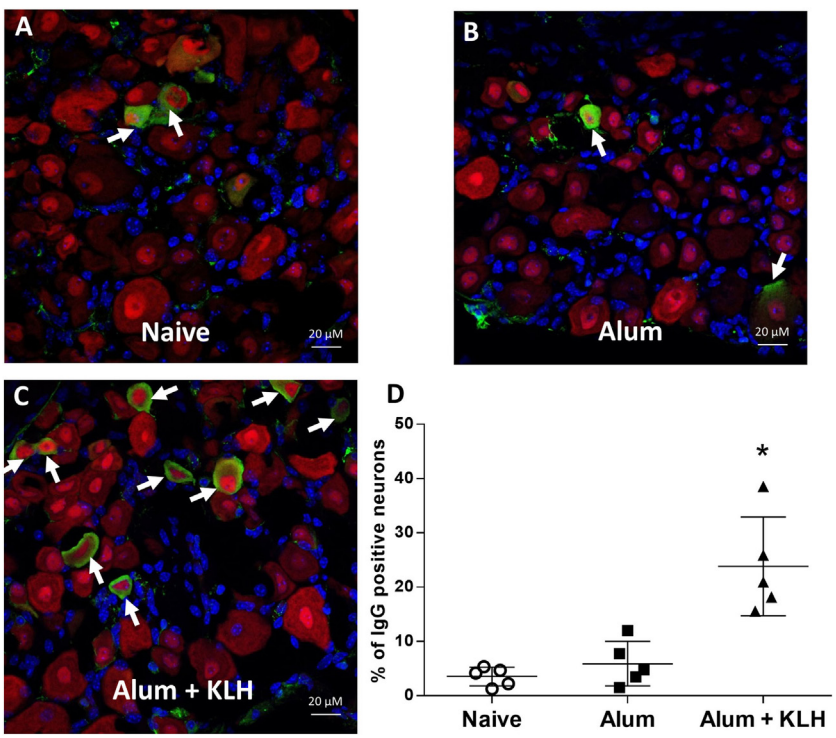

FIGURE 1 | Sensory neurons of the mouse dorsal root ganglia (DRGs) exhibit abundant IgG reactivity. Immunohistochemical staining of whole DRGs isolated from **(A)** naïve, **(B)** alum-injected, and **(C)** alum + KLH-immunized C57BL/6J mice to detect neurons (NeuN, red), IgG (green), and nuclei (DAPI, blue). 400× magnification; scale bar = 20 μm. **(D)** Percentage of double IgG-positive, NeuN-positive neurons (among total NeuN-positive neurons) in DRGs isolated from naïve, alum-injected, and alum + KLH-immunized mice ($n = 5$ per group). A total number of 824, 1,343, and 814 NeuN+ DRG neurons were assessed for immunostaining for the naïve, alum-treated and alum + KLH-immunized mice, respectively. Data are shown as mean percentage ± SD. *$P < 0.05$ vs naïve DRGs Kruskal–Wallis one way ANOVA (followed by Dunn's Multiple Comparison Test).

the neuronal nucleus, and IgG protein is primarily distributed in the cytoplasm of sensory neurons (**Figures 1A–C**).

Sensory Neurons From DRG Release Antigen-Specific IgG_1

To investigate the antigen-specificity of antibodies sequestered in neurons, DRG sensory neurons were cultured on ELISpot plates. A significant increase in the number of total IgG-releasing neurons was observed in DRGs isolated from alum + KLH-immunized mice as compared to alum-injected controls (**Figures 2A,B**). It is interesting to note that fewer neurons releasing IgG were detected in animals that received only alum (**Figures 2A,B**), which is in agreement with our immunohistochemical data showing low numbers of sensory neurons sequestering antibodies in DRGs of alum-injected controls (**Figure 1B**). Similar to alum + KLH-immunization, a significant increase in number of neurons releasing total IgG and OVA-specific IgG was observed in DRGs harvested from alum + OVA-immunized mice (**Figures 2C,D**). As alum + KLH-immunized mice exhibited robust responses, we utilized this model for our subsequent studies. The majority of these DRG sensory neurons from alum + KLH-immunized mice released IgG_1 isotype (**Figure 2E**). To study the antigen specificity of the IgG associated with DRG sensory neurons, we next assessed the number of IgG-releasing neurons in a KLH-specific or an OVA-specific ELISpot assay. A significant increase in

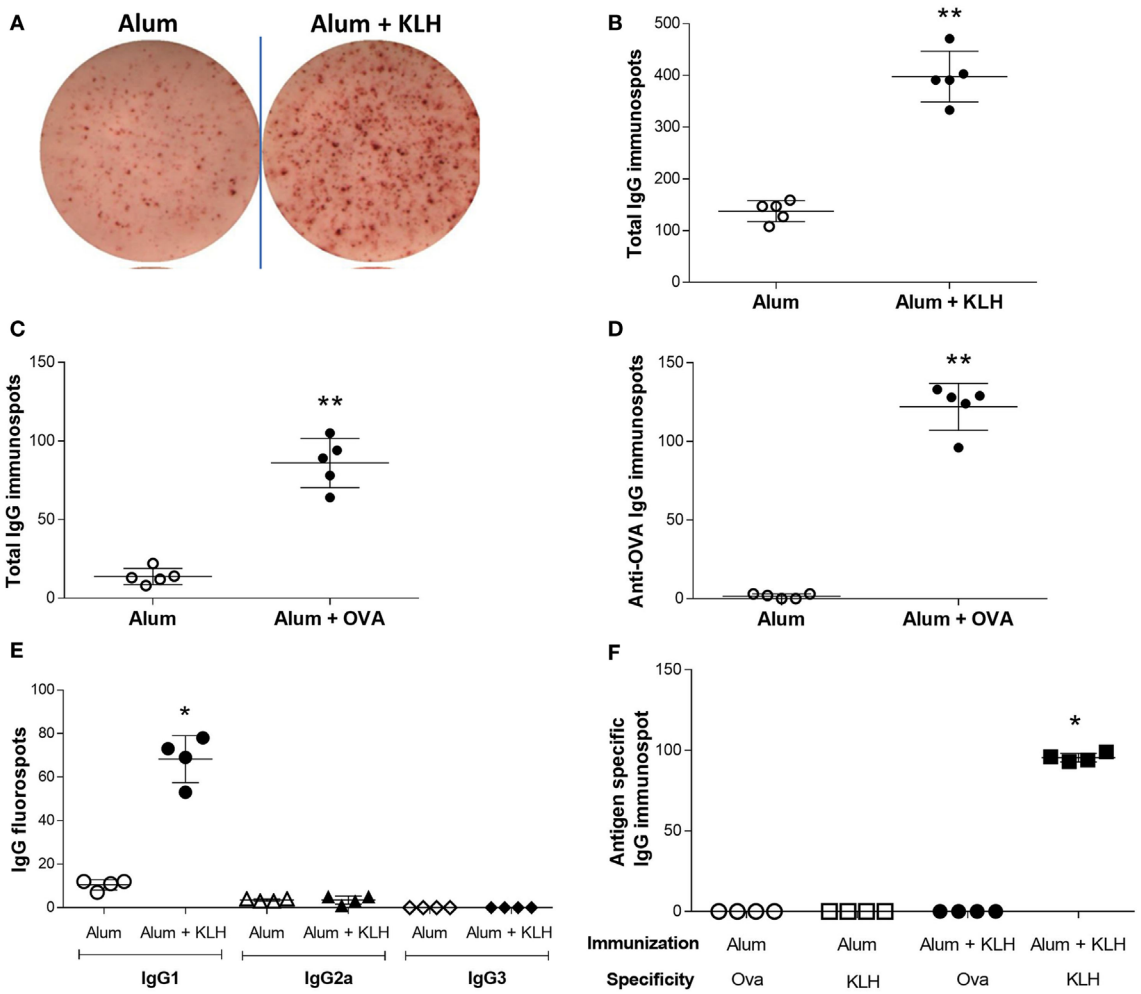

FIGURE 2 | Dorsal root ganglion (DRG) neurons release antigen-specific IgG_1. **(A)** Representative images from total IgG ELISpot assay performed on DRG neurons isolated from alum-injected and alum + KLH-immunized mice. Quantitation of total IgG-releasing DRG neurons isolated from **(B)** alum-injected and alum + KLH-immunized mice ($n = 5$ per group) or **(C)** alum-injected and alum + ovalbumin (OVA)-immunized mice ($n = 5$ per group) using the ELISpot assay (mean ± SD); $^{**}P < 0.01$ *vs*. alum-injected (Mann-Whiteny test). **(D)** Quantitation of anti-OVA-releasing DRG neurons isolated from alum-injected and alum + OVA-immunized mice ($n = 5$ per group) using the Anti-OVA IgG ELISpot assay (mean ± SD); $^{**}P < 0.01$ vs. alum-injected (Mann-Whitney test). **(E)** Quantification of IgG_1-, IgG_{2a}-, and IgG_3-specific FluoroSpots from DRG neurons isolated from alum-injected and alum + KLH-immunized mice using the IgG isotype FluoroSpot assay; (mean ± SD, $n = 4$ per group); $^{*}P < 0.05$ *vs*. alum-injected (Mann-Whitney test). **(F)** Quantification of anti-OVA- and anti-KLH-specific IgG immunospots from DRG neurons isolated from alum-injected and alum + KLH-immunized mice (mean ± SD, $n = 4$ per group) using the antigen-specific ELISpot assay; $^{*}P < 0.05$ *vs*. alum-injected for each antigen (Mann-Whitney test).

KLH-specific neurons was observed in animals immunized with alum + KLH as compared to alum-injected controls (**Figure 2F**). In contrast, no anti-OVA IgG-releasing neurons were detected in DRGs isolated from either alum-injected or alum + KLH-immunized mice (**Figure 2F**), indicating that antigen-specific IgG antibodies are associated with DRG sensory neurons in immunized animals.

DRG Sensory Neurons Lack Expression of Essential Enzymes Required for IgG Synthesis

To assess if sensory neurons from DRGs have the molecular machinery to synthesize antibodies and undergo class-switching, we analyzed the transcriptome of the neuronal population using RNA-seq. DRGs were harvested from animals immunized with alum + KLH (n = 10) or alum-injected controls (n = 10) and RNA-seq analysis was performed using the RNA prepared from pooled DRG neurons for downstream processing to maximize biological diversity. The RNA-seq analysis was primarily used to evaluate the overall transcriptome of the DRG neuron preparations and not to statistically compare the alum vs. alum + KLH-immunized groups. As an exploratory step for characterizing the DRG neuron preparations, RNA-seq was performed using the Illumina NextSeq platform. An average of 17,355 and 17,030 genes were detected (detection > 3 transcripts per million, TPM) in the two groups studied, alum-injected and alum + KLH-immunized, respectively.

Further examination of the RNA-seq data revealed that the expression of neuronal and nociceptor-related gene expression was present in the alum-injected and alum + KLH-immunized groups (**Table 2**). In contrast, the expression of B lymphocyte, T lymphocyte, mast cell, and basophil-related genes were not observed in either of the groups. Both neuronal preparations show a similar low level of positive regulatory domain zinc finger protein-1 (*Prdm1*) expression (**Table 2**), which encodes Blimp1, a protein required for the differentiation of B cells into plasma cells (20). These data demonstrate that the enriched neuronal preparation in our study is devoid of B lymphocytes. We next evaluated whether the DRG sensory neuron populations express any of the essential genes required for antibody synthesis and class-switching. No expression of *Rag1, Rag2, Aicda,* or *Ung* genes was observed in either alum-injected or alum + KLH-immunized group (**Table 2**), indicating that DRG sensory neurons lack the genetic make-up to produce antibodies.

To confirm the RNA-seq data, we validated the expression of genes required for antibody synthesis and B cell differentiation into plasmablasts/plasma cells at different time points post-immunization. DRG neuronal preparations were isolated from animals (alum-injected controls or alum + KLH-immunized) at three different time points (n = 10 mice per group): post-primary immunization, post-first booster and post-second booster as described in the Section "Materials and Methods." The expression pattern of four genes related to antibody synthesis (*Rag1, Rag2, Aicda,* and *Ung*) and two genes corresponding to B lymphocyte/plasma cell markers (*Cd19* and *Prdm1*) were validated by quantitative real-time RT-PCR. As expected, high levels of the B cell marker, *Cd19*, and low-moderate levels of *Rag1, Rag2, Aicda,* and *Ung* were observed in the naïve spleen. However, DRG sensory neurons failed to express *Rag1, Aicda* or *Ung* at any of the time points studied (**Table 3**). Our preliminary RNA-seq data confirmed the expression of *Il4ra* (the gene that encodes the alpha chain of the IL-4 receptor, a type I transmembrane protein that can bind IL-4 and IL-13 to regulate IgG production) in DRG sensory neurons (TPM value ~5) and the expression of the *Tlr4* gene (that encodes the receptor for LPS) (TPM value ~7). To address the possibility that the molecular events related to antibody synthesis are not operative during the DRG sample collection and analysis, but may be induced in a similar manner as activated B cells, we stimulated DRG sensory neurons (or splenic B cells as a positive control) *in vitro* and assessed the expression of *Rag2* and *Aicda* at three different time points post-stimulation. We used two conditions for *in vitro* activation of DRG sensory neurons and B cells: LPS + IL-4 (T-independent) or anti-CD40 + IL-4 (T-dependent) and no treatment (vehicle). As reported previously (21, 22), stimulation of mature mouse B cells with LPS + IL-4 resulted in the expression of both *Rag2* and *Aicda in vitro* in a time-dependent manner (**Figure 3**). In contrast, activation of DRG sensory neurons with either T-independent (LPS + IL-4) or T-dependent (anti-CD40 + IL-4) stimuli failed to induce either *Rag2* or *Aicda* gene expression (**Figure 3**). These data confirm that DRG sensory neurons lack the expression of essential enzymes required for IgG synthesis.

Transgenic *Ighg1*-tdTomato Mice Do Not Express tdTomato in DRG Sensory Neurons

Casola et al (23) have produced a transgenic *Ighg1*-cre mouse that can be used in combination with a reporter gene to analyze the transcription of Igγ1 constant region gene segment (Cγ1) in antibody expressing cells. We utilized the *Ighg1*-cre mice to induce expression of a fluorescent reporter protein, tdTomato, in antibody-expressing cells because IgG_1 was found to be the most prominent subtype in the DRG neurons (**Figure 2E**). The elimination of the STOP cassette and subsequent tdTomato expression was achieved by crossing *Ighg1*-cre mice with B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J mice, resulting in tdTomato expression mostly in cells expressing Cγ1 and hence, IgG_1. Transgenic mice (carrying both cre and tdTomato) were indistinguishable from their WT littermate controls (carrying only cre or tdTomato) in terms of developmental features. tdTomato is one of the brightest among the red-fluorescent proteins and well-suited for immunohistochemical analysis. Accordingly, we found that the tdTomato reporter mice had increased numbers of tdTomato + cells in their spleens following immunization (**Figure 4A**). However, no colocalization of tdTomato fluorescent signal and antibody staining was observed in sensory neurons following immunization (**Figure 4B**). As tdTomato expression is driven by the transcription of Cγ1 (of IgG_1), these data confirm that *Ighg1* are not expressed in sensory neurons from DRG.

TABLE 2 | Expression of neuron, non-neuron, Fc gamma receptor, and antibody recombination gene expression by DRGs.

Neuronal marker genes	RefSeq ID	Protein	Alum-TPM	Alum + KLH -TPM
Nes	NM_016701	Nestin	22.40	22.48
Nefh	NM_010904	Neurofilament Heavy, NF200	1,020.71	973.48
Nefl	NM_010910	Neurofilament Light, NF68	5,259.31	5,048.04
Nefm	NM_008691	Neurofilament Medium, NF3	2,262.77	2,181.69
Rbfox3	NM_001285438	NeuN	54.34	37.77
Nociceptor-related genes				
Nrp2	NM_008737	Neuropilin 2	91.98	94.94
Scn1a	NM_018733	$Na_v1.1$	140.59	161.37
Scn7a	NM_009135	$Na_v2.1$	693.59	694.48
Scn10a	NM_009134	$Na_v1.8$	293.78	276.91
Scn11a	NM_011887	$Na_v1.9$	374.29	330.16
Trpa1	NM_177781	TRPA1	153.92	130.88
Trpv1	NM_001001445	TRPV1	72.59	73.55
Trpv2	NM_011706	TRPV2	10.51	7.61
B cell marker genes				
Cd5	NM_007650	CD5	0	0
Cd19	NM_009844	CD19	0	0
Cd22	NM_009845	CD22	0.79	0.76
Ptprc	NM_011210	B220; CD45R	0.44	0
Prdm1	NM_007548	BLIMP1	3.79	3.07
T cell marker genes				
Cd3e	NM 007648	CD3e	0.19	0.04
Cd3g	NM 009850	CD3y	0	0
Cd8a	NM_001081110	CD8A	0	0
Cd80	NM_009855	CD80	0.89	0.93
Basophil and mast cell genes				
Cd200r3	NM_029018	CD200R3	0	0
Cd200r3	NM_001128132	CD200R3	0	0
Cd200r3	NM_027578	CD200R3	0	0
Anpep	NM_008486	CD13	0.13	0.16
Il3ra	NM_008369	IL-3Ra	0.19	0.04
Fc gamma receptor genes				
Fcgr1	NM_010186	FcyR1	0.07	0.04
Fcgr2b	NM 010187	FcyR2b	5.67	4.44
Fcgr2b	NM 001077189	FcyR2b	0	0.66
Fcgr3	NM_010188	FcyR3	0.3	0.3
Fcgr4	NM_144559	FcyR4	4.32	3.97
Fcgrt	NM_010189	FcRn	2.31	1.40
Ab recombination genes				
Aicda	NM_009645	AID	0.05	0.02
Rag1	NM_009019	RAG1	0.08	0.06
Rag2	NM_009020	RAG2	0.61	0.56
Ung	NM_011677	Uracil-DNA glycosylase (UNG)	1.27	0.71

For RNA-Seq: RNA was isolated from DRG neurons collected from alum-injected mice and alum + KLH-immunized mice (n = 10 animals per group – pooled). The values expressed are TPM values for each pooled sample (without SDs). TPM, transcripts per million (relative abundance of gene expression based on the length of each gene and for sequencing depth).

DRG Sensory Neurons Lacking Neuronal *Rag2* Expression Contain Antigen-Specific Antibodies

Because neuronal *Rag2* expression has been implicated in previous work (24–27), here we crossed floxed-*Rag2* transgenic mice with synapsin-cre transgenic mice generating null mutants that selectively lack *Rag2* expression in neurons (with abundant *Rag2* expression in B cells). Analysis of immunoglobulin reactivity in the DRGs of null mutants revealed the presence of IgG-positive NeuN-positive neurons in both alum-injected (**Figure 5A**) and alum + KLH-immunized (**Figure 5B**) mice. Similar to previous observations with WT mice (**Figures 2A,B,F**), significantly higher numbers of IgG-releasing and anti-KLH IgG-releasing DRG neurons were observed in the alum + KLH group when compared to the alum-injected group even in the absence of *Rag2* expression (**Figures 5C,D**). Taken together, our results indicate that sensory neurons from DRGs lack the enzymes and cannot synthesize antibodies.

Antibodies Found in DRG Neurons Are Released by B Cells

Accordingly, we reasoned that the neurons from DRGs sequester and retain antibodies produced by plasma cells, and therefore, DRGs isolated from mice incapable of producing B cell-derived

TABLE 3 | *In vivo* analysis of gene expression by Q-PCR.

	Day 0		Day 14		Day 28			
	Alum	Alum + KLH	Alum	Alum + KLH	Alum	Alum + KLH	Naïve DRG	Naïve Spleen
Rag1	1.0 ± 0.2	2.0 ± 0.2	0.6 ± 0.4	0.6 ± 0.3	1.2 ± 0.4	0.7 ± 0.2	0.7 ± 0.2	41.05 ± 5.3
Rag2	1.0 ± 0.4	1.3 ± 0.6	12.2 ± 3.1	57.4 ± 9.9	18.0 ± 1.6	43.4 ± 14.7	1.8 ± 0.4	2,083 ± 370
Aicda	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	750 ± 149
Ung	1.0 ± 0.1	1.5 ± 0.2	0.7 ± 0.2	1.1 ± 0.2	1.4 ± 0.1	1.3 ± 0.3	0.8 ± 0.1	21.7 ± 1.3
CD19	1.0 ± 0.0	0.4 ± 0.2	0.8 ± 0.0	1.2 ± 0.0	1.8 ± 0.4	1.6 ± 1.7	8.9 ± 2.5	5,7300 ± 8,261
Prdm1	1.0 ± 0.1	1.1 ± 0.2	0.3 ± 0.0	0.4 ± 0.0	0.9 ± 0.2	0.7 ± 0.2	0.3 ± 0.0	14.7 ± 1.8

Mice (n = 3 per group) were injected with either alum alone or alum + KLH on days 0, 14, and 28; DRG neurons were isolated 24 h after the last injection as described in the Section "Materials and Methods." RNA was isolated from resultant DRG neurons, as well as from DRG neurons and spleens harvested from naïve mice. Gene expression was analyzed by qPCR. Data are shown as relative fold changes (mean ± SD) normalized to alum-injected group. Results reveal the lack of Rag1, Aicda, and Ung in DRG neurons across time points and the presence of gene expression in the Naïve spleen.

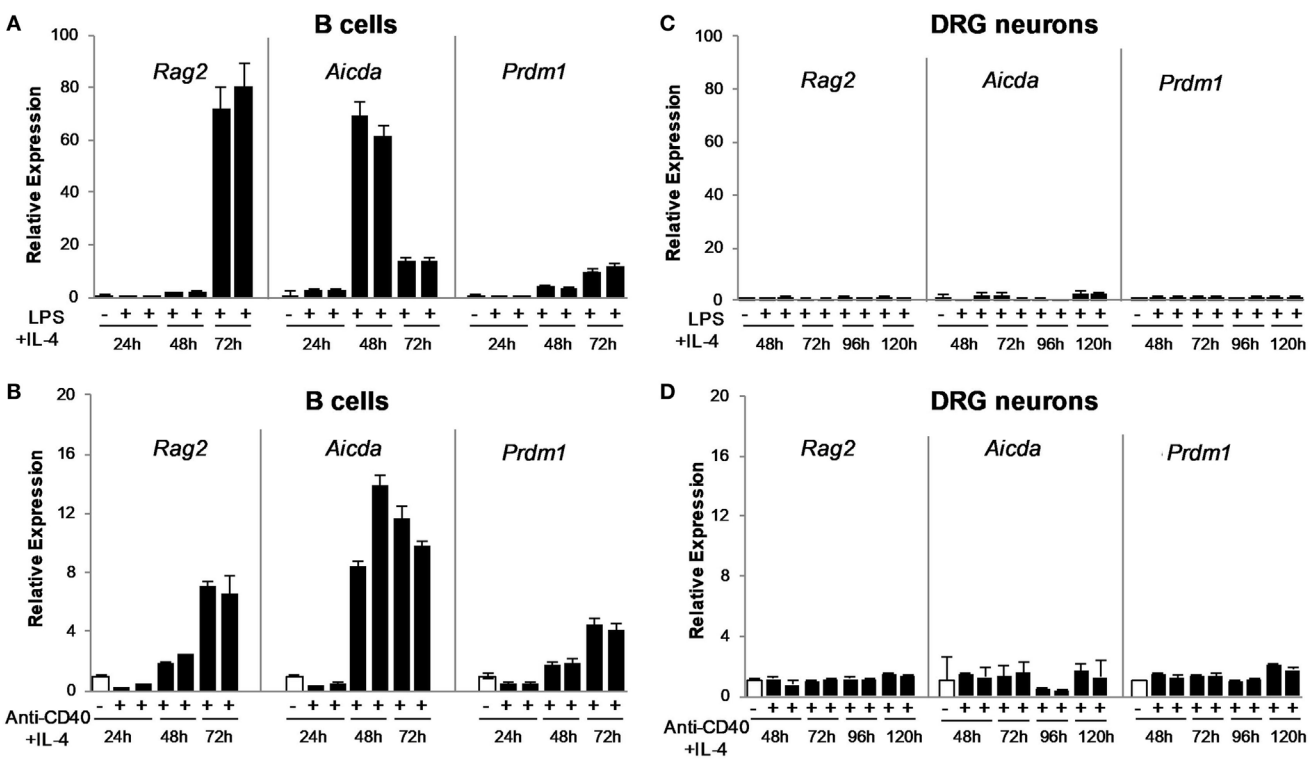

FIGURE 3 | *In vitro* stimulated splenic B cells, but not dorsal root ganglion (DRG) sensory neurons, express B cell activation markers associated with antibody synthesis. Naïve splenic B cells stimulated *in vitro* for 24–72 h with LPS + IL-4 **(A)** or anti-CD40 + IL-4 **(B)** express genes associated with antibody production (*Rag2* and *Aicda*), as well as *Prdm1*, a marker of activated B cells/plasma cells. Stimulated B cells are compared to naïve freshly isolated, untreated B cells. In contrast, DRG neurons stimulated *in vitro* with LPS + IL-4 **(C)** and anti-CD40 + IL-4 **(D)** for up to 120 h do not exhibit gene expression associated with antibody production (*Rag2* and *Aicda*) or *Prdm1* when compared to untreated DRG neurons. Stimulated DRG neurons are compared to vehicle-treated cells at each time point. Data are shown as mean ± SD quantitative PCR (qPCR) data for duplicate samples.

antibodies would contain no antigen-specific antibodies. To address this question, we utilized mice with a homozygous targeted disruption of the membrane exon of the Ig mu-chain (μMT mice) that are deficient of mature peripheral B cells as well as *Rag1* KO mice that do not produce mature T and B lymphocytes (28–30). Immunohistochemical analysis revealed that DRGs from either μMT or *Rag1* KO mice do not contain antigen-specific antibodies (**Figures 6A,B**). Next, we generated chimeric mice by transferring *Rag1* KO BM to WT mice or WT BM to *Rag1* KO mice. After confirming the reconstitution of the recipient animals (data not shown), the chimeric mice received alum injections or alum + KLH immunizations as described. Immunohistochemical analysis of chimeric mice with *Rag1*-deficient BM (*Rag1*-negative hematopoietic cells, but *Rag1*-positive DRG sensory

neurons) revealed that in the absence of antibody secreting plasma cells, no IgG reactivity is observed in sensory neurons from DRGs isolated from either alum-injected (**Figure 7A**) or alum + KLH-immunized groups (**Figures 7B,C**). In contrast, IgG-positive neuronal populations were observed in DRGs from alum-injected chimeric mice with WT BM (*Rag1*-positive hematopoietic cells, *Rag1*-negative sensory neurons) (**Figure 7D**). Furthermore, immunization with alum + KLH significantly increased the number of IgG-positive sensory neurons in the DRGs as observed with WT mice (**Figures 7E,F**).

We next analyzed the immunoglobulin release by DRG sensory neurons from chimeric mice using both total IgG and KLH-specific IgG ELISpot assays. Increased IgG-positive neuronal cells are observed in the DRGs harvested from chimeric mice with WT BM, but not in the chimeric mice with *Rag1*-deficient BM (**Figure 8A**). Analysis of KLH-specific IgG-releasing cells revealed a significant increase in anti-KLH IgG-positive DRG sensory neurons in the DRGs isolated from alum + KLH-immunized chimeric mice with WT BM (**Figure 8B**). In contrast, no anti-KLH IgG-secreting DRG sensory neurons are observed in immunized chimeric mice with *Rag1*-deficient BM (**Figure 8B**). Administration of alum alone also increased the number of total IgG-releasing DRG sensory neurons, but not anti-KLH IgG-positive neurons (**Figures 8A,B**). Together, these data suggest that DRG sensory neurons not only sequester and retain antibodies secreted by plasma B cells but also can release these antibodies.

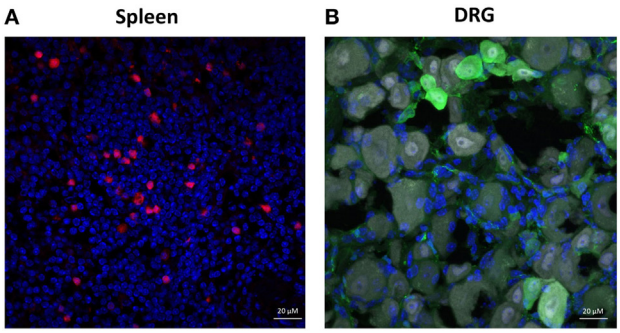

FIGURE 4 | Mouse dorsal root ganglion (DRG) neurons lack Igγ1 chain expression. Immunohistochemical staining of spleen and whole DRGs isolated from alum + KLH-immunized mice expressing tdTomato under the control of the *Ighg1* promoter. **(A)** Spleens express Igγ1 chain (red) (DAPI = blue). **(B)** DRG neurons express NeuN (gray) and IgG (green), but not Igγ1 chain (red; tdTomato) (DAPI = blue). 400× magnification; scale bar = 20 μm.

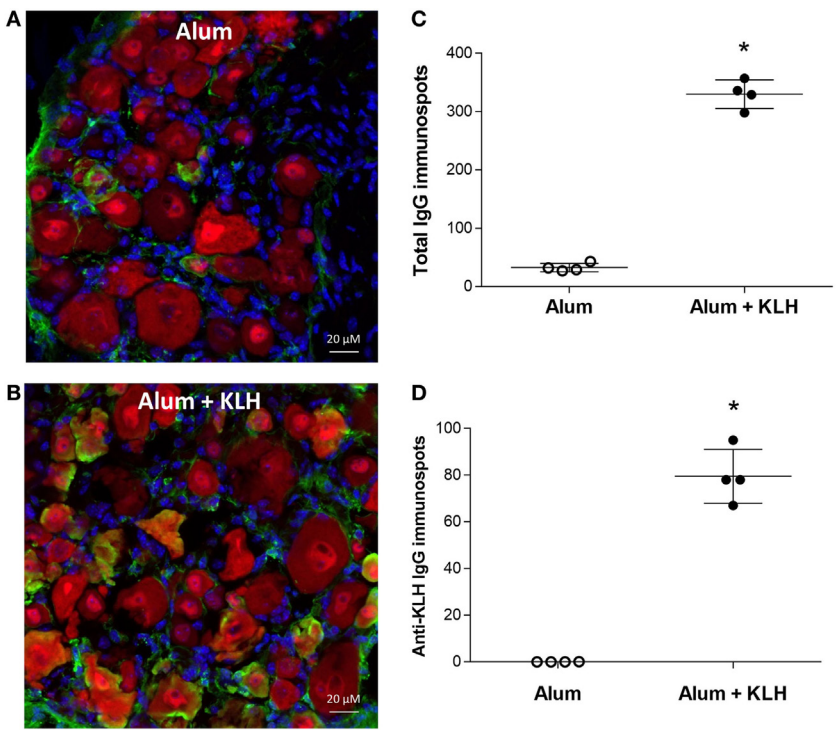

FIGURE 5 | Dorsal root ganglion (DRG) neurons from mice lacking neuronal-specific *Rag2* expression contain antigen-specific antibodies. Immunohistochemical staining of whole DRGs isolated from alum-injected **(A)** and alum + KLH-immunized **(B)** neuronal–specific *Rag2* knock-out (KO) mice for IgG (green), neurons (NeuN, red), and nuclei (DAPI, blue). 400× magnification; scale bar = 20 μm. **(C)** Quantitation of IgG-releasing DRG neurons isolated from alum-injected and alum + KLH-immunized neuronal-specific *Rag2* KO mice using the ELISpot assay (mean ± SD, $n = 4$ per group); $^*P < 0.05$ *vs*. alum-injected (Mann-Whitney test). **(D)** Quantification of anti-KLH-specific IgG immunospots from DRG neurons isolated from alum-injected and alum + KLH-immunized neuronal-specific *Rag2* KO mice (mean ± SD, $n = 4$ per group) using the antigen-specific ELISpot assay $^*p < 0.05$ *vs*. alum-injected (Mann-Whitney test).

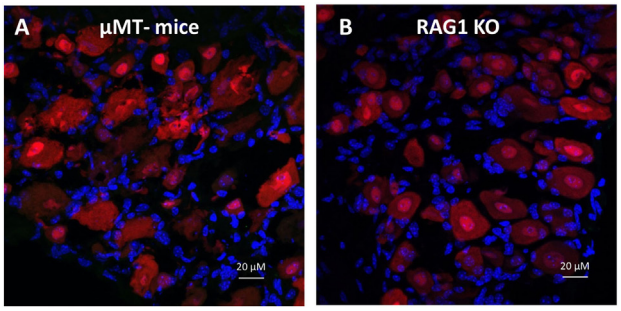

FIGURE 6 | Dorsal root ganglion (DRG) neurons from mice deficient in B cell-derived IgGs [μMt and *Rag1* knock out (KO)] lack antigen-specific IgG immunoreactivity. Immunohistochemical staining of whole DRGs isolated from alum + KLH-immunized μMt **(A)** and *Rag1* KO **(B)** mice stained for neurons (NeuN, red), IgG (green), and nuclei (DAPI, blue), 400× magnification; scale bar = 20 μm.

DISCUSSION

Here we demonstrate that antigen-specific antibodies are sequestered in mouse DRG sensory neurons. A significantly higher number of DRG sensory neurons from immunized animals showed sequestration of antigen-specific antibodies as compared to alum-injected control or naïve mice. RNA-seq analysis and qPCR of the neuronal transcripts revealed that sensory neurons from DRGs lack the genetic machinery to produce antibodies. Using transgenic and chimeric animals, we further confirmed that DRG sensory neurons do not synthesize antigen-specific antibodies, but sequester the antibodies released by plasma cells.

As shown in **Figure 1**, DRG neurons isolated from naive mice and alum-injected mice had similar low percentages of IgG-containing NeuN+ neurons (~4–6%), while DRG neurons isolated from mice immunized with alum + KLH showed significantly higher percentages (~24%). Antibody localization appears

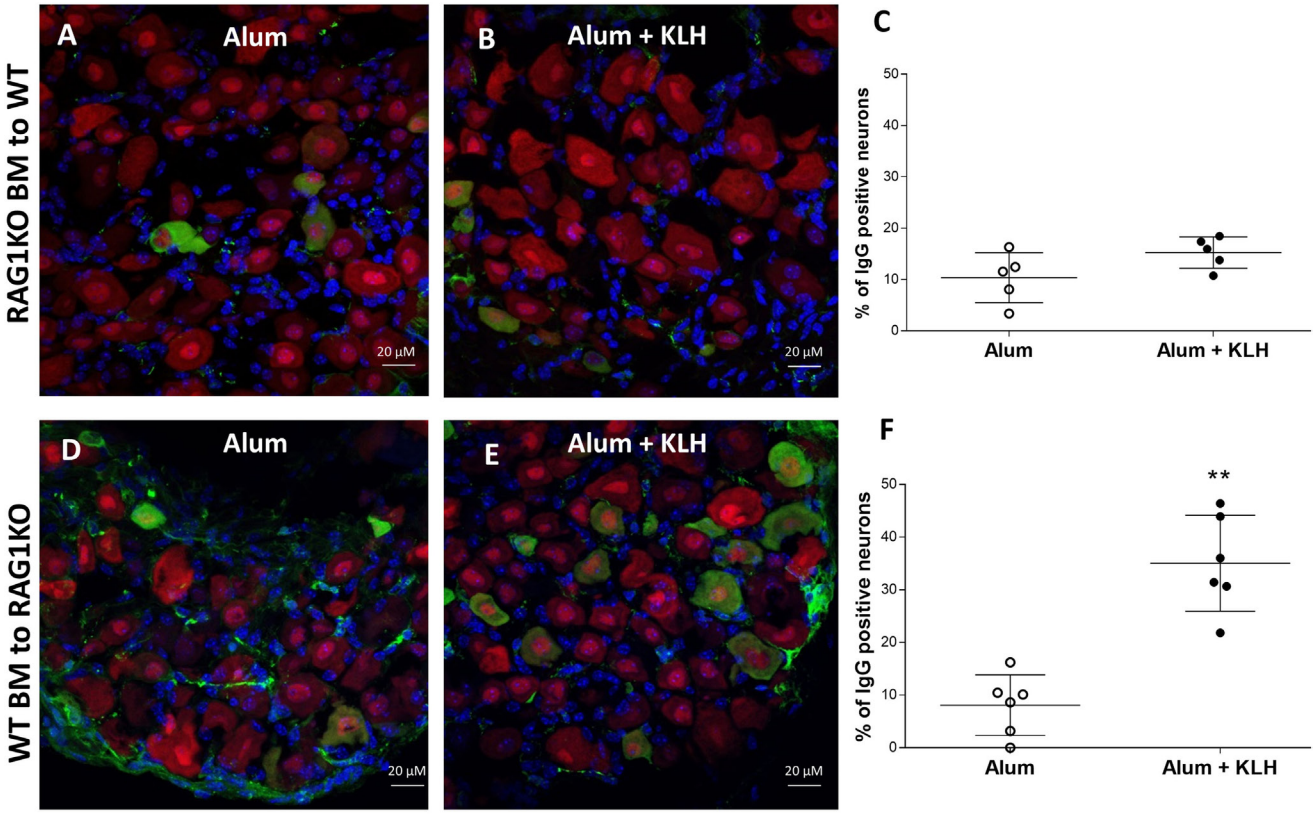

FIGURE 7 | Dorsal root ganglion (DRG) neurons from chimeric mice with *Rag1* knock out (KO) BM lack immunization-induced antibody accumulation. Immunostaining of whole DRGs isolated from alum-injected **(A)** and alum + KLH-immunized **(B)** chimeric mice (*Rag1* KO BM to WT) for neurons (NeuN, red), IgG (green), and nuclei (DAPI, blue). 400× magnification; scale bar = 20 μm. **(C)** Percentage of double IgG-positive, NeuN-positive neurons (among total NeuN-positive neurons) in DRGs from isolated from alum-injected and alum + KLH-immunized chimeric mice (*Rag1* KO BM into WT mice); data are shown as mean percentage ± SD; ($n = 5$ per group). Immunostaining of whole DRGs isolated from alum-injected **(D)** and alum + KLH-immunized **(E)** chimeric mice (WT BM into *Rag1* KO) for neurons (NeuN, red), IgG (green), and nuclei (DAPI, blue). 400× magnification; scale bar = 20 μm. **(F)** Percentage of double IgG-positive, NeuN-positive neurons (among total NeuN-positive neurons) in DRGs isolated from alum-injected and alum + KLH-immunized chimeric mice (WT BM to *Rag1* KO); (mean percentage ± SD, $n = 6$ per group). $^{**}P < 0.05$ *vs*. alum-injected (Mann-Whitney test).

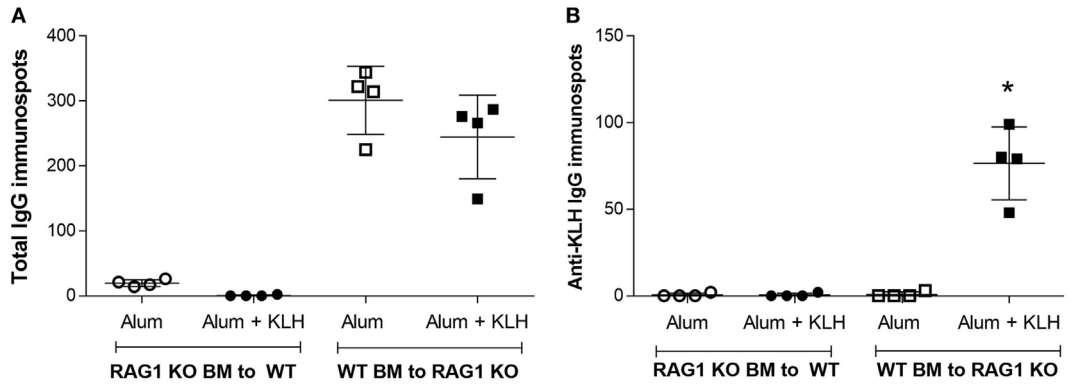

FIGURE 8 | Dorsal root ganglion (DRG) sensory neurons from mice with *Rag1*-positive hematopoietic cells, but not from mice with *Rag1*-negative hematopoietic cells, release antigen-specific IgG. Detection of IgG-releasing cells using the ELISpot assay. Quantification of **(A)** total IgG and **(B)** anti-KLH-specific IgG immunospots produced by DRG neurons isolated from alum-injected and alum + KLH-immunized chimeric mice [*Rag1* knock-out (KO) bone marrow (BM) into wild-type (WT) and WT BM into *Rag1* KO]. (Mean ± SD, $n = 4$ per group). *$P < 0.05$ *vs*. alum-injected (Mann-Whitney test).

intracellular. However, the subcellular localization of IgG within NeuN+ DRG neurons remains to be determined. All additional experiments included the alum-injected and alum + KLH-immunized groups. Although this study design limited our ability to compare DRG neurons obtained from naive *vs*. alum-treated mice, it focused our studies on assessing antigen-specific antibody responses in alum-injected vs. alum + KLH-immunized mice. The anti-KLH antibodies released by DRG neurons were primarily IgG1 (not IgG2 or IgG3) (**Figure 2D**). We did not assay for IgA, IgE, or IgM antibodies. Future studies will examine what stimuli (e.g., antigen, capsaicin, or KCl) trigger IgG release by DRG neurons.

Antibodies are normally synthesized by mature plasma cells. However, recent accumulating evidence suggests that non-lymphoid cells, such as human sperm (31), hepatocytes (32), certain carcinoma cells (25, 32, 33), and neurons from the central nervous system (34–37), also express genes required for antibody synthesis and produce antibodies. Here, we demonstrate that although DRG sensory neurons exhibit localization of antigen-specific antibodies, the neuronal cells do not express the essential enzymes required for generating antibody diversity. V(D)J recombination is tightly regulated during B cell differentiation by transcriptional regulation of lymphoid-specific recombinase genes encoding RAG1 and RAG2 enzymes (38–41). In mature B cells, antigenic stimulation induces somatic hypermutation, gene conversion, and class switch recombination leading to generation of a secondary repertoire of high-affinity antigen-specific antibodies (42, 43). Activation-induced cytidine deaminase (AID), an enzyme encoded by the *Aicda* gene, is responsible for regulating and coordinating these three genetic rearrangements in mature B cells (42, 44). AID generates antibody diversity by converting cytosine to uracil within the immunoglobulin loci resulting in mismatch mutations. The uracil-guanine mismatch created by AID is repaired by uracil-DNA glycosylase (UNG), a DNA-repair enzyme encoded by the gene *Ung,* introducing base-pair changes resulting in antibody diversity (42). The DRG neuronal transcriptome analysis here revealed the absence of *Rag1, Rag2, Aicda,* and *Ung* transcripts. Furthermore, *in vitro* activation of DRG sensory neurons with LPS and IL-4 (to induce T-independent class switch recombination) or anti-CD40 and IL-4 (to induce T-dependent class switch recombination) did not induce significant increases in expression of either *Rag1, Rag2*, *Aicda,* or *Ung* genes. Moreover, a comprehensive transcriptome analysis of 622 hand-picked single neurons harvested from lumbar DRGs of naïve mice showed no detectable *Rag1, Rag2,* or *Aicda* expression (16).

An interesting finding in the current study is the marked discordance in the expression of *Rag1* and *Rag2* transcripts in DRG sensory neurons harvested from immunized mice. Amplification of the neuronal transcripts showed low levels of *Rag2* but no *Rag1* transcripts present in DRG sensory neurons. It is unlikely that DNA contamination contributed to the results, as all samples were treated with DNase before analysis, and intron spanning primers were used. These data rather suggest that *Rag1* and *Rag2* are differentially expressed in immunized mice. As discussed earlier, expression of *Rag1* and *Rag2* is tightly controlled and occurs primarily during the early developmental stages of T and B cells. A *cis*-regulatory element located in the RAG locus regulates coordinated expression of *Rag1* and *Rag2*. However, a less-strict control of transcription within the RAG locus results in functionless expression of *Rag2* transcript in the brain and other non-lymphoid tissues (27). Although expression of *Rag1* and *Rag2* transcripts have been reported in a variety of non-lymphoid cells, including different cancer cell types, epithelial cells and neurons (34, 45, 46), to our knowledge, no functional role for these enzymes have been identified in non-lymphoid cells.

In order to investigate the functional role of the RAG2 enzyme for antibody synthesis in neurons, we developed a neuron-specific *Rag2* KO mouse to detect if antibodies are sequestered in neurons in the absence of neuronal *Rag2* gene expression. We immunized the neuronal *Rag2* KO mice with alum + KLH and

observed a significant increase in antibody sequestration, total IgG and anti-KLH IgG-releasing neurons in DRGs from immunized mice even in the absence of *Rag2* expression in neurons. Mice deficient in either RAG1 or RAG2 enzymes lack mature lymphocytes and fail to generate antibodies due to their inability to initiate V(D)J rearrangement (30, 47). In the absence of V(D)J recombination and mature B cells, *Rag1* KO mice completely lacked antibody localization in their DRG sensory neurons. Consistent with this observation, no localization of antibodies was observed in mice homozygous for an inactivating mutation of the membrane exon of their immunoglobulin μ chain gene (μMT) that results in mature B cell deficiency and no antibody production (29). Adoptive transfer of WT BM into *Rag1*-deficient mice restored antibody sequestration in DRGs. Together, these results indicate that DRG sensory neurons sequester antibodies released by mature B cells.

Antibody accumulation has been described in brain tissues and cerebrospinal fluid in both healthy and pathological conditions (35, 37, 48, 49). Antigen-antibody immune complexes directly activate sensory neurons resulting in an increase in intracellular calcium (9, 50), initiation of action potentials (8, 50), and the release of neurotransmitters from DRG neurons (9). The presence of antibodies in the nervous system could be attributed to receptor-mediated uptake of antibodies by neurons. Immunoglobulin G and its receptors have been observed in the human nervous system (34), rodent brains (9, 35, 37, 51), and zebrafish sensory neurons (24, 46). Expression of different subtypes of Fcγ receptors have been shown in Purkinje cells, parvalbumin neurons, primary neuronal cultures, and sensory neurons (52–54). While RNA-seq and qPCR (data not shown) failed to detect the expression of either *Fcgr1* or *Fcgr3* in DRG sensory neurons, the expression of one *Fcgr2b* variant and *Fcgrt* were detected (**Table 2**). Consistent with our data, single cell analysis of the DRG neuron transcriptome revealed a lack of expression of Fcγ receptors 1 and 3 (16). However, the presence of FcRn, an MHC class I related antibody receptor, has been detected in sensory neurons (16). FcRn, a product of the *Fcgrt* gene, plays a pivotal role in passively transferring IgG within and across multiple cell types (55–59) and protecting IgG from degradation (59–61). Mice genetically deficient in *Fcgrt* (lacking FcRn) have significantly reduced plasma half-life of IgG and exhibit hypogammaglobulinemia (60–62). In the central nervous system, FcRn expressed by endothelial cells has been implicated in antibody transport across the blood brain barrier (63). Similar to the situation in the central nervous system, it is possible that in DRGs, FcRn mediates the transport of antibodies into sensory neurons and protects the IgGs from degradation within the neuronal cells. Further studies are required to address the functions of Fcγ receptors and FcRn in peripheral DRG sensory neurons. Future studies using *in vitro* and *in vivo* methods including single cell qPCR (to compare neurons that sequester IgG *vs*. those that do not) will provide insight on how and why antigen-specific antibodies are taken up by DRG neurons.

Several possibilities exist that may explain the accumulation of antibodies in sensory neurons. Antibody uptake by neurons may have a self-protecting effect. Using a murine stroke model, Arumugam et al have demonstrated that intravenously injected IgG protects the brain against neuronal death by neutralizing the effects of complement (64). In a mouse model of Alzheimer's disease, antibodies assisted clearance of amyloid β-peptide from the CNS into the circulation *via* FcRn (65). Antigen-specific antibodies also aid in clearance of neuronal-specific pathogens from neurons. Treatment of encephalitis virus-infected primary cultured neurons with virus-specific antibodies clears the infectious encephalitis virus from neurons by restricting viral gene expression (66). Accordingly, immunodeficient *scid* mice infected with the encephalitis virus develop persistent non-lethal central nervous system infection (66). It is also possible that invading pathogens get opsonized by the locally released antibodies and are subsequently phagocytosed by glial cells (67). In addition, antigen-antibody complexes may mediate hyperalgesia by activating peripheral sensory neurons. Numerous antigen-specific immune-related disorders are often accompanied by pain. These disorders include autoimmune diseases such as rheumatoid arthritis (68) and Guillain-Barre syndrome (69), viral reactivation syndromes such as herpes zoster (70, 71), and allergic diseases such as atopic and allergic contact dermatitis (72, 73). Elevated levels of antigen-specific antibodies, especially IgG, and immune complexes are found in the serum and affected tissues in these diseases. Immune complexes directly activate nociceptive DRG neurons in a TRPC3-receptor dependent manner, and increase neuronal excitability and thus, potentially contribute to pain (7, 8). Recent studies have demonstrated that intradermal injection of immune complexes dose-dependently produces mechanical and thermal hyperalgesia in the hind paws of rats that can be alleviated by localized administration of non-specific antibody (74). Finally, it is important to note that our studies were confined to DRG sensory neurons. We did not study other sensory neurons (e.g., nodose or trigeminal ganglia).

Our studies demonstrate that peripheral DRG sensory neurons sequester antibodies made by plasma cells. In concluding that DRG neurons do not produce antibodies we are at risk of a type II error (in failing to reject a null hypothesis that is actually false). We have attempted to reduce the risk of this error by analyzing large numbers of DRG neurons from the mice and by testing the same hypothesis using multiple approaches and models [qPCR, RNA-seq, immunostaining, and both *in vitro* and *in vivo* models (using multiple genetically modified mice and BM transfer techniques)]. Therefore, we are confident that our results demonstrate that DRG neurons sequester antigen-specific antibodies. These antigen-specific antibodies may play an important role in protecting DRG neurons from pathogen-induced damage, or in inducing antigen-specific hyperalgesia. These findings, together with the previous studies, implicate immune complexes in mediating neuro-immune crosstalk between DRG sensory neurons and antibodies in both physiological and pathological conditions.

ETHICS STATEMENT

All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols

approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) of the Feinstein Institute for Medical Research, Northwell Health, Manhasset, NY, USA.

AUTHOR CONTRIBUTIONS

MKG, CNM, KJT, and SSC designed research; MKG, PKC, AS, GHI, MA, GK, AL, CNM and SSC performed research; MKG, PKC, AS, GK, JFG, DM, JA, UA, TRC, CNM, KJT, and SSC analyzed and interpreted data; MKG, TRC, CNM, KJT and SSC wrote the article; MM, CP, MAC, TWM, CB, BS and BD provided additional comments and contributed to finalizing the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00638/full#supplementary-material.

FIGURE S1 | Cellular composition of DRG neuron preparations. The principal DRG cell types in the sensory neuron preparations isolated from DRGs collected from alum-injected and alum + KLH-immunized mice include neuronal cells: peptidergic nociceptor (PEP) cells, non-peptidergic nociceptor (NP) cells, and neurofilament containing (NF) cells, and non-neuronal (NoN) cells. The cell types were defined by Usoskin and colleagues (16), and the relative proportions were computed using RNA-seq deconvolution analyses, as described in the Section "Materials and Methods."

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REVIEW
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The Cholinergic System Modulates Memory and Hippocampal Plasticity *via* Its Interactions with Non-Neuronal Cells

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Degeneration of central cholinergic neurons impairs memory, and enhancement of cholinergic synapses improves cognitive processes. Cholinergic signaling is also anti-inflammatory, and neuroinflammation is increasingly linked to adverse memory, especially in Alzheimer's disease. Much of the evidence surrounding cholinergic impacts on the neuroimmune system focuses on the α7 nicotinic acetylcholine (ACh) receptor, as stimulation of this receptor prevents many of the effects of immune activation. Microglia and astrocytes both express this receptor, so it is possible that some cholinergic effects may be *via* these non-neuronal cells. Though the presence of microglia is required for memory, overactivated microglia due to an immune challenge overproduce inflammatory cytokines, which is adverse for memory. Blocking these exaggerated effects, specifically by decreasing the release of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6), has been shown to prevent inflammation-induced memory impairment. While there is considerable evidence that cholinergic signaling improves memory, fewer studies have linked the "cholinergic anti-inflammatory pathway" to memory processes. This review will summarize the current understanding of the cholinergic anti-inflammatory pathway as it relates to memory and will argue that one mechanism by which the cholinergic system modulates hippocampal memory processes is its influence on neuroimmune function *via* the α7 nicotinic ACh receptor.

Keywords: alpha 7 nicotinic acetylcholine receptor, hippocampal memory, microglia, cholinergic anti-inflammatory pathway, neuroinflammation

INTRODUCTION

Cholinergic circuits have been implicated in normal and abnormal cognitive functioning since 1906, when Dr. Aloysius Alzheimer described the symptomatology and neuropathology of the disease that today bears his name (1). Disruption of cholinergic circuitry is likely to be at least partly responsible for the cognitive impairments seen in neurodegenerative disorders (2, 3). Recent studies have also revealed deficits in cholinergic signaling in disorders of attention and cognitive control [see Ballinger et al. (4)]. While the mechanism by which cholinergic signaling influences cognitive processes has been assumed to be direct cholinergic stimulation of pre- and postsynaptic neuronal receptors, a neglected area of investigation is the role of acetylcholine's (ACh) peripheral and central anti-inflammatory effects on cognition. Neuroinflammation is also a hallmark

of neurodegenerative disorders and has been implicated in the neuropathogenesis of Alzheimer's disease (AD). Not only do neurons respond directly to ACh but also do non-neuronal cells: peripheral macrophages, as well as microglia and astrocytes in the central nervous system (CNS). These non-neuronal cells influence short-term and long-term synaptic function and plasticity [reviewed in Achour and Pascual (5)], and through these mechanisms may contribute to both dysfunction and improvements in cognition.

Though certainly not the only mechanism, this review posits that ACh may influence hippocampal function *via* peripheral and central immune cells, and through this intermediary process, may alter neuronal processes underlying cognition (6). First, this review will describe the intercellular components and pathways of the cholinergic system relevant to memory, with a special focus on the effects of ACh in the hippocampus. Second, this review will explore the role of ACh in hippocampal memory and plasticity, examining both the direct and indirect roles that ACh may have in modulating hippocampal function. A final section of the review will highlight how cholinergic modulation of the immune system may provide new perspectives on regulating memory dysfunction in disease.

THE CHOLINERGIC SYSTEM

Synthesis and Synapses

Acetylcholine was first identified by Dale (7) for its actions on heart tissue. It was later recognized as a neurotransmitter by Loewi (8), who initially named it "Vagusstoff" because it was released from the vagus nerve. Since then, the intricate workings of ACh synthesis and synaptic communication have been identified.

Cholinergic synthesis and reuptake in neurons is well understood. First, ACh is synthesized from choline and acetyl-CoA *via* the choline acetyltransferase (ChAT) enzyme. ACh is subsequently transported into vesicles and released into the synaptic cleft, where it can bind to the muscarinic and/or nicotinic ACh receptors. Within the synapse, ACh is broken back down into choline and acetic acid by acetylcholinesterase (AChE). Choline reuptake occurs *via* a high affinity choline transporter, and then choline is recycled in the synthesis of new ACh.

However, neurons are not the only cells to synthesize ACh: cells from the skin, kidney, eye, liver, and placenta all contain ChAT (9). T-cells also show ChAT activity, synthesize ACh, and have been shown to "relay the neural signal" in the cholinergic anti-inflammatory pathway by releasing ACh, which subsequently acts on macrophages *via* the α7 nicotinic ACh receptor (10). ChAT activity has also been found in non-neuronal cells in the CNS, specifically in astrocytes (11). At this time, it is not clear whether microglia show any ChAT activity (12). Further work is needed to pinpoint the cell types involved in ACh synthesis and how they act upon and with neuronal ACh.

Receptors

There are two kinds of ACh receptors: nicotinic (nAChR) and muscarinic (mAChR). nAChRs, which will be a focus of this review, are ligand-gated ion channels and occur in the neuromuscular junction, autonomic ganglia, and throughout the CNS. One specific subtype of nAChR identified to be functionally important in hippocampal memory [though not the only one; see Chan et al. (13)] is the α7 nAChR.

Using various agonists and antagonists such as nicotine and α-bungarotoxin, nAChRs have been extensively mapped in the rodent brain (14), and, to a lesser extent, the human brain (15). Notably, the hippocampus has almost every nAChR subtype (16), has a high density of α7 nAChR receptors, and expresses cholinergic receptors both pre- and post-synaptically (17). The distribution of receptors is highly preserved across species and is similar in both rodent and human brains (15).

As reviewed in Albuquerque et al. (6), nAChRs are also present in non-neuronal cells, including keratinocytes, endothelial cells, cells in the digestive, respiratory, and peripheral immune systems, and—critically—on glia (18–20). In the brain, both microglia (21, 22) and astrocytes (23) express α7 nAChRs (22, 24).

Alpha-bungarotoxin, which is a specific antagonist to α7 and α9 nAChRs, was used to show the dense nAChR population on human macrophage surfaces (25). Additionally, administration of nicotine decreased α-bungarotoxin binding, further providing support for the specificity of this marker. RT-PCR, western blotting, α-bungarotoxin-conjugated beads, and cloning of cDNA showed definitively that the α7 nAChR was specifically responsible for this binding (25).

Circuits

In the CNS, cholinergic neurons reside in three major areas: (1) there are cholinergic neurons in the brainstem, where they may function in risk aversion (26). The cholinergic neurons in this area project to and inhibit the thalamus (27, 28). (2) There are cholinergic interneurons in the striatum, which suppress dopamine release (29). (3) There are cholinergic neurons that originate in the basal forebrain, mainly in the medial septum, vertical limb of the diagonal band (MS/VDB), horizontal limbs of the diagonal band, and nucleus basalis. These cells project to the olfactory bulb, neocortex, hippocampus, and amygdala (30–32). Cholinergic neurons in the MS/VDB project to all of the subregions of the hippocampus (33, 34). There are also cholinergic interneurons in the cortex itself, but they are scarce (35). Basal forebrain neurons, specifically those in the nucleus basalis, selectively degenerate in AD (36) and have been a focus of research on the relation between ACh and memory.

There are also projections from the basal forebrain to the frontal cortex, which are involved in attentional processes (37). Attention is known to have a beneficial role in memory itself. Though the interaction of attention and memory is beyond the scope of this review, it is important to note that both functions rely on cholinergic projections from the basal forebrain, so experiments manipulating these connections could be impacting both memory and attention.

Interestingly, few of the recent reviews (38–42) of ACh actions in the CNS mention that many non-neuronal cells in the body and brain manufacture and respond to ACh. As well, few acknowledge that peripheral ACh actions may impact CNS function. These findings are reviewed below.

ACh in the Periphery

Acetylcholine is the neurotransmitter in all preganglionic neurons in both the sympathetic and parasympathetic nervous systems, as well as all parasympathetic postganglionic neurons. However, only a small number of sympathetic postganglionic neurons are cholinergic (those innervating sweat glands) whereas the rest are adrenergic. Within the somatic nervous system, all motor neurons that innervate skeletal muscles are cholinergic.

The Cholinergic Anti-inflammatory Pathway

The finding that activation of the efferent vagus nerve inhibits proinflammatory cytokine release and protects against peripheral inflammation led to this connection between ACh and inflammation to be named "the cholinergic anti-inflammatory pathway" [(43); see **Figure 1**]. Stimulation of the vagus nerve, either endogenously or through electrical stimulation, leads to increased ACh release (synthesized from T-cells), which acts on macrophage α7 nAChRs. This activation leads to a decreased production of inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6), by macrophages [reviewed in Gallowitsch-Puerta and Pavlov (44)]. Peripheral ACh also acts on α7 nAChRs on lymphocytes to suppress inflammation (20, 45–47). ACh also produces a dose-dependent inhibition of IL-6, IL-1β, and TNF-α production in human macrophages [reviewed in Pavlov and Tracey (43) and Borovikova et al. (48)] and in whole-blood of rheumatoid arthritis patients (49), whereas vagotomy leads to increases in IL-6 and, to a lesser extent, TNF-α (48).

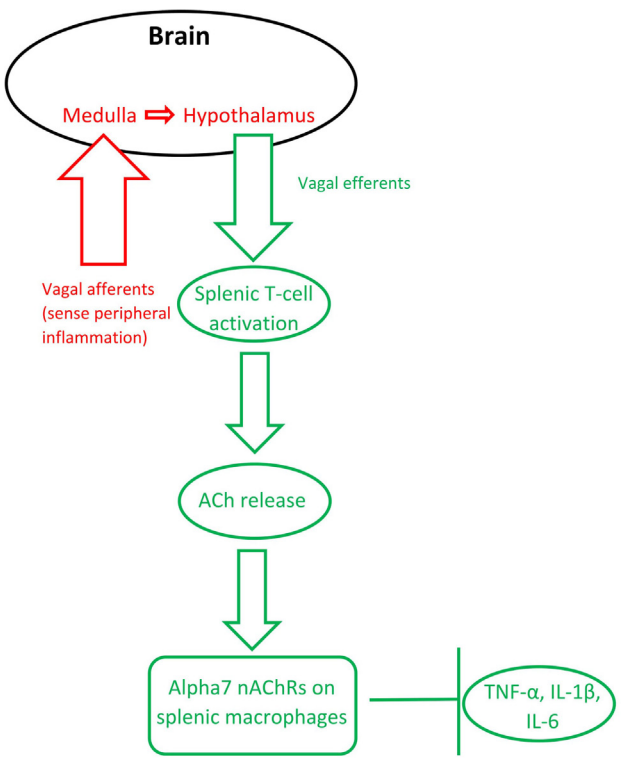

FIGURE 1 | The inflammatory reflex (red) and the cholinergic anti-inflammatory pathway (green). Based on Steinberg et al. (50) and Gallowitsch-Puerta and Pavlov (44).

Importantly, the vagus nerve's anti-inflammatory action is also bidirectional: the afferent vagus nerve detects peripheral cytokines, then communicates through the medulla to the hypothalamus, which subsequently communicates *via* the efferent vagus nerve to inhibit inflammation in the periphery (51). In other words, the bidirectional anti-inflammatory communication between the brain and periphery relies on the vagus nerve and ACh signaling. Termed the "inflammatory reflex," the afferent vagus immediately regulates production of pro-inflammatory cytokines to avoid overproduction (52).

The cholinergic anti-inflammatory pathway relies on activation of α7 nAChRs on macrophages. Alpha7 nAChR stimulation has been shown to modulate TNF-α release (53). AChE inhibitor administration, which decreases the breakdown of ACh, leads to lower levels of IL-6 and TNF-α, but does not significantly alter cytokine release in α7 nAChR knockout mice (54). Macrophages stimulated with the bacterial mimic lipopolysaccharide (LPS) produce more TNF-α, which is blunted by nicotine pretreatment; however, additional pretreatment with an antisense oligonucleotide for α7 nAChRs (but not α1 or α10 nAChRs) ameliorated this effect (25). These findings specifically implicate the α7 nAChR, a receptor otherwise known to be important for hippocampal memory, in the cholinergic anti-inflammatory pathway.

Like peripheral macrophages, microglia have α7 nAChRs, which, when activated, suppress pro-inflammatory cytokine release (53, 55). In mouse and human cell culture studies, it has been shown that α7 nAChRs on microglia are necessary for blunting TNF-α and downstream IL-1β production (55, 56). AChE inhibitors have been shown to suppress TNF-α secretion from microglia, and addition of α-bungarotoxin blunted these effects (57). Peripheral macrophages and microglia—the macrophages of the brain—seem to respond similarly to ACh.

Thus far, this review has summarized evidence that ACh is synthesized by neurons but also by non-neuronal cells. Basal forebrain cholinergic neurons release ACh in all regions of the hippocampus, which all contain nAChRs. The hippocampus in particular is a region with a high density of microglia and astrocytes (58) as well as a high density of nAChRs. These findings suggest that cholinergic stimulation of the hippocampus not only has direct neuronal effects but also effects on microglia and astrocytes that may modulate neuronal function.

MEMORY AND NEURAL PLASTICITY

The Classic View of the Cholinergic System in Memory

Recent reviews of basal forebrain cholinergic systems in memory and cognition (4, 59–62) focus on the septohippocampal pathway, which is widely known to be implicated in memory processes. Below, the standard view of septohippocampal ACh functions in memory are reviewed, followed by a proposal for some alternate means by which ACh may have effects on hippocampal memory.

There are a number of lines of evidence that support the view that hippocampal ACh is important for memory [see Parent and

Baxter (63)]. First, during spatial memory tasks, cholinergic markers such as ChAT are upregulated [see Park et al. (64)]. Second, ACh levels in the hippocampus are correlated with memory function. For example, there is a correlation between age-related cognitive decline and decreases in hippocampal ACh (65). Multiple studies find a correlation between spatial memory and ACh release both in the hippocampus (66), and within the basal forebrain (67). Also, damage to the septum leads to decreases in both spatial memory performance and hippocampal levels of ACh (68). Function can be rescued when basal forebrain AChE is inhibited pharmacologically. Third, both the direct infusion of ACh into the hippocampus and direct pharmacological activation of nAChRs in the hippocampus reverse the cognitive deficits caused by damage to the septum (69–72). Importantly, this finding shows that while the basal forebrain provides multiple inputs to the hippocampus, direct activation of nAChRs in the hippocampus reverses cognitive dysfunction caused by interruption of this pathway. While all of these data have traditionally been interpreted as direct actions of ACh on neuronal receptors, hippocampal astrocytes (18) and microglia (53) also express nAChRs. Therefore, all of these findings leave open the possibility that some of the actions of ACh on the hippocampus may be *via* nicotinic activation of glial cells. However, more research to tease apart the direct neuronal and indirect non-neuronal actions of ACh in memory is needed.

ACh is the "Decider" between Encoding and Retrieval

The classically held view is that ACh is the decider between encoding mode and retrieval mode in memory processing (73, 74). ACh is associated with suppressing old associations and inhibiting proactive interference. Rats with cholinergic basal forebrain lesions perform comparably to controls in a water maze task unless the location of the platform changed daily (75). An explanation for this finding is that the lack of ACh in the hippocampus leads to more expression of a previously encoded association (which would be the previous location of the platform). However, rats with intact cholinergic systems are able to inhibit the previous association, and form a new one. Hasselmo (74) in addition to Easton et al. (73) provided extensive reviews describing what may be the neural underpinnings of this phenomenon. Briefly, the CA1 region of the hippocampus receives input from two brain regions: entorhinal cortex layer 3 (associated with sensory perception—"extrinsic input") and the CA3 region of the hippocampus (associated with previously formed associations—"intrinsic input"). ACh reduces the relative input from CA3, hence allowing sensory inputs to be encoded, free from proactive inhibition. In this way, hippocampal ACh "prioritizes encoding" in novel contexts. This extended model implicates hippocampal ACh directly in the encoding phase and also allows working memory to be more efficient.

Further support for this hypothesis mostly relies on hippocampal ACh and its association with increased exploratory behavior. Evidence shows that hippocampal ACh levels are increased in instances of novelty (76) and exploratory rearing (77) compared to non-novel environments. The authors conclude from this work that the state of novelty itself activates the cholinergic system. Frontal cortical and dorsal hippocampal ACh, glutamate, and GABA measured through microdialysis in response to exploration of a novel environment support this interpretation (76). In both brain areas, but exaggerated in the hippocampus, ACh was significantly increased during the first session of exploration. Additionally, infusion of ACh agonists into the hippocampus led to increased exploratory behavior (78). The take-away from these studies is that increased hippocampal ACh release, caused by a novel environment, aids encoding and increases exploratory behavior. While this evidence points to hippocampal ACh as an indicator and facilitator of encoding in novel contexts, it does not address the contribution of other cells that ACh may stimulate.

Non-Neuronal Actions of the Cholinergic System in Memory

Recent work has, in fact, demonstrated that ACh acts directly on hippocampal astrocytes, which then leads to alterations in firing of hippocampal neurons (79). Consistent with Hasselmo's view (80) that high levels of ACh aid encoding by suppressing inappropriate activations, specific optogenetic stimulation of septal cholinergic neurons led to decreased firing of dentate granule cells. Administration of an α7 nAChR antagonist blocked this effect, indicating that this receptor is responsible for the decreased firing. The critical aspect of the findings, however, is that disrupting astrocytic function in the hilar layer of the hippocampus by an inhibitor of glial metabolism prevented inhibition of dentate granule cells caused by septal cholinergic stimulation. These findings revealed that septohippocampal release of ACh causes a slow inhibition of dentate granule cells, not by acting directly on neurons but rather by activating astrocytes (see **Figure 2**). As well, it was found that a nAChR antagonist prevented activation of astrocytes, indicating that astrocytes are specifically responding to basal forebrain cholinergic inputs through this class of receptor, specifically the α7 nAChR. When activated, astrocytes can release neurotransmitters like glutamate (81, 82) and consequently suppress activation of dentate granule cells *via* hilar inhibitory interneurons. As further support for the view that hippocampal astrocytes play a role in hippocampal function, blocking transmitter release from astrocytes impaired hippocampal-dependent novel object recognition memory, but not all types of memory (83). These data provide support for the view that basal forebrain ACh release may influence hippocampus and memory *via* slow inhibition of neuronal activity *via* astrocyte intermediaries.

In addition to astrocytes, microglia are necessary for hippocampal memory and motor-learning dependent synapse formation, likely *via* microglial brain-derived neurotrophic factor (BDNF) (84). To address whether or not microglia are necessary for memory, mice expressing tamoxifen-inducible Cre recombinase that allowed for specific manipulation of gene function in microglia were generated. Then, Cre was used to induce diphtheria toxin receptor expression solely in microglia. Mice depleted of microglia *via* diphtheria toxin administration showed deficits in hippocampal-dependent fear conditioning and novel object-memory tasks, indicating that microglia are critical for emotional and hippocampal-dependent memory (85). As well, depleting

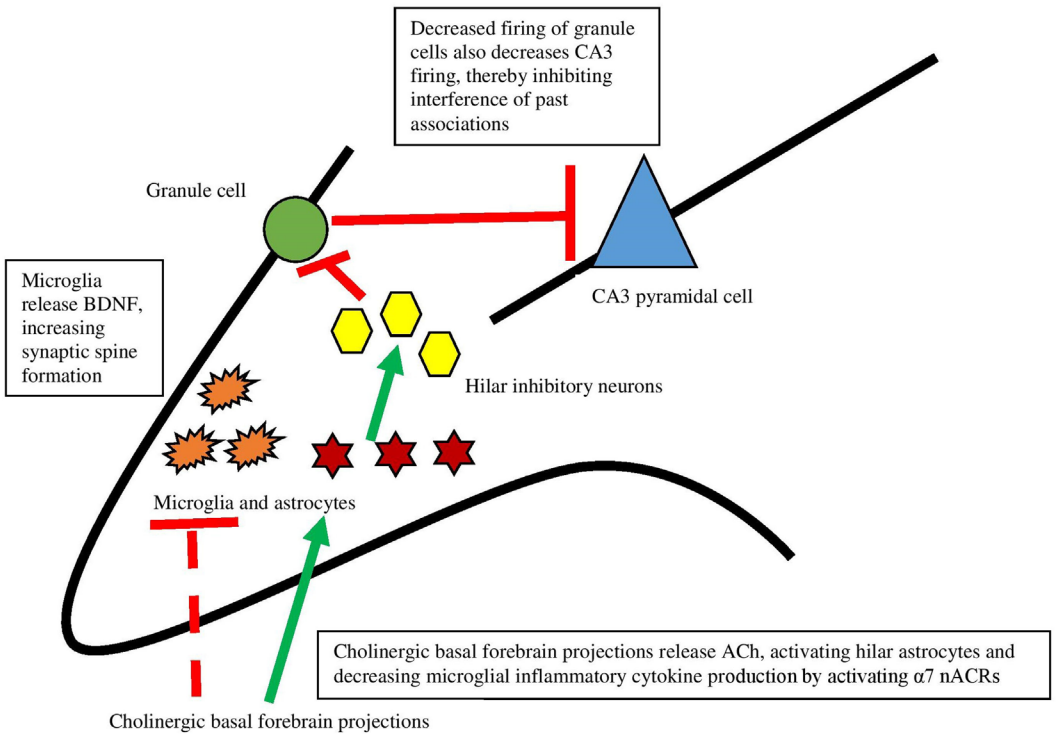

FIGURE 2 | The interaction between the cholinergic system and glia and its impact on the hippocampus. Dotted lines represent cytokine effects, and solid lines represent cell activation/firing. Cholinergic basal forebrain projections release ACh and both decrease cytokine release from microglia and activate hilar astrocytes. Those astrocytes activate inhibitory interneurons, which decreases firing from granule cells. This decreased firing leads to decreased firing of CA3 pyramidal cells, preventing interference of past associations in encoding. Based largely on Hasselmo (74) and Pabst et al. (79).

microglial BDNF mimicked the effects of microglial elimination on memory function and synaptic spine formation, suggesting a potential mechanism for the memory effects. Interestingly, there were no differences in the expression of TNF-α, IL-1β, and IL-6 in microglial-depleted and control mice—possibly indicating a compensatory production mechanism (for example, production of cytokines by non-microglial cells like astrocytes, or cytokines entering the CNS through the blood–brain barrier). Regardless, the memory impairments were not due to the increase or decrease of these cytokines. Microglial depletion has also been shown to impair spatial memory in mice trained on a Barnes maze (86).

Though microglia are necessary for memory, overactivation of microglia, which causes increased release of proinflammatory cytokines (such as TNF-α, IL-1β, and IL-6), is detrimental. This has been demonstrated in several studies of postoperative cognitive dysfunction (POCD).

Postoperative cognitive dysfunction is a decline in memory and executive functions that occurs shortly following surgery in some patients and can persist for several months or more (87). Memory impairment also occurs following peripheral orthopedic surgery in mice (88, 89). This mouse model of POCD has revealed that surgery not only increases pro-inflammatory cytokines in the periphery but also upregulates inflammation in the brain (90). These effects are not due to anesthesia, as mice given anesthesia without surgery do not show this inflammatory profile (90). Macrophage-produced TNF-α appears to promote POCD by altering the permeability of the blood-brain barrier, allowing increased macrophage infiltration to the hippocampus after orthopedic surgery (88): prevention of macrophage-produced TNF-α prevented the increased permeability of the blood–brain barrier, and subsequently also prevented the increased macrophage migration into the brain after surgery. Orthopedic surgery leads to increased IL-1β in the hippocampus and impaired hippocampal-dependent fear conditioning, while anti-TNF antibody administration inhibits this effect (90).

Interestingly, stimulation of α7 nAChRs prevents the macrophage migration and cognitive deficits seen after surgery, and administration of an α7 antagonist increased neuroinflammation and POCD (88, 89). Because one of the agonists, choline, does not easily cross the blood–brain barrier, the authors conclude that it must be acting on peripheral macrophages. The cholinergic anti-inflammatory pathway takes effect both peripherally by acting on macrophages to decrease proinflammatory cytokine release, and centrally by decreasing hippocampal pro-inflammatory cytokines (89) and macrophage activation and migration. Both of these inflammatory metrics influence fear conditioning: surgery leads to impaired memory, but α7 agonists rescue this behavior. Alpha7 antagonists further impair memory after surgery. Notably, orthopedic surgery also decreases hippocampal BDNF and neurogenesis (91).

In a study examining the effect of minocycline administration and surgery on inflammation and memory in an aged-mouse

model of POCD, minocycline (which decreases microglial activation, but may also have effects on neurons) administered prior to surgery not only decreases hippocampal levels of inflammatory cytokines TNF-α, IL-1β, and interferon-γ but also rescues spatial memory deficits that occur following surgery (92). Previous findings showing that microglia have α7 nAChRs suggest that activation of the cholinergic anti-inflammatory pathway might have similar effects to treatment with minocycline. The authors suggest that microglia may "get stuck" in an activated state after an immune challenge, and the continued release of inflammatory cytokines might facilitate cognitive aging and the associated memory impairment.

There is considerable support for the important role of cytokines released from microglia in memory modulation. Administration of inflammatory cytokines (specifically, TNF-α and IL-1β) causes deficits in spatial memory (93–95) and hippocampal-dependent fear conditioning [(96); for review, see Pugh et al. (97)]. Following infection, it appears that it is microglia, not neurons or astrocytes, in the hippocampus that are responsible for the increase in IL-1β, which then leads to impaired memory (98). Blocking IL-1β release in the CNS prevents the memory impairment caused by overactivation of microglia (99). Importantly, the complete absence of IL-1β is detrimental to memory—indicating basal levels are necessary, but overexpression of the cytokine is harmful (98). Even in the absence of other inflammatory factors and insults, the increased expression of TNF-α led to decreased performance in memory tasks such as passive avoidance (100). Though there is much research on the impact of inflammation in memory, more work is needed on the integration of ACh and neuroimmune factors on hippocampal-dependent memory.

Markers of Plasticity: Neuronal and Non-Neuronal Influences

As described previously, basal forebrain cholinergic inputs to the hippocampus play an important role in cognitive function. Therefore, it is not surprising that there is considerable evidence that ACh receptors are involved in various aspects of neural plasticity: long-term potentiation (LTP), regulation of BDNF, and hippocampal neurogenesis.

Long-term Potentiation

Acetylcholine "biases the system" toward increased LTP, believed to be one of the cellular foundations of learning and memory, by decreasing the induction threshold required (101, 102). In addition, in an *in vitro* high ACh environment, stimulation that normally produces long-term depression produces LTP (103). The specific neuronal mechanisms underlying this effect have largely been identified. ACh, when it binds to a muscarinic ACh receptor, leads to a signaling cascade activating phospholipase-C, which has been shown to contribute to LTP (104). Additionally, impaired LTP has been linked to malfunctioning α7 nAChRs (105). A blockade of α7 nAChRs blunted LTP, and α7 nAChR knockout mice can similarly show decreased LTP (106). The cholinergic systems' impact on LTP has always been interpreted as a direct synaptic action, but it is also possible that ACh is acting on glial α7 nAChRs, though research to date has not demonstrated this conclusively.

Impairments in hippocampal LTP have been linked to microglial overactivation, and minocycline normalizes these impairments (107). Clearly, microglia have an important role in LTP that has not been explored fully, and this role is possibly mediated by microglial α7 nAChRs.

Pharmacological activation of α7 nAChRs leads to increases in hippocampal LTP, quantified by long-lasting increases in calcium activity in the CA1 and CA3 regions of the hippocampus in wild-type, but not α7 nAChR knockout mice (108). Because the α7 nAChR is highly permeable to calcium, this specific receptor is likely causal for this effect (109). These data support the view that α7 nAChR activation on neurons as well as on microglia both aid LTP.

The effect of α7 nAChR activation is usually the decreased release of inflammatory cytokines, and these molecules have also been shown to impact LTP. TNF-α directly modulates the strength of synapses by altering postsynaptic AMPA receptor expression (110), leading to weakened synaptic strength and increased likelihood for that synapse to be engulfed by activated microglia. A proposed mechanism that may explain cognitive dysfunction in patients with immune disorders is suppression of the cholinergic anti-inflammatory pathway leading to heightened secretion of TNF-α, altering astrocyte-neuron signaling, leading to a cascade resulting in a restructuring of the excitability of hippocampal synapses (111).

During LTP and hippocampal memory tasks, hippocampal IL-1β is released, and blocking IL-1 receptors has an adverse effect on both memory and LTP (98, 112). So, though IL-1β is required for LTP, overexpression—such as that seen in pathological conditions—inhibits LTP (113). In other words, the effects of IL-1β on LTP follow the same "U-shaped" concentration-response curve as the effects of IL-1β in memory: moderate levels are necessary, but overexpression is detrimental. Because ACh inhibits release of inflammatory cytokines, perhaps the impact of ACh on LTP occurs *via* both direct neuronal and indirect non-neuronal action.

Brain-Derived Neurotrophic Factor

Acetylcholine has been shown to modulate plasticity *via* BDNF. Following chronic nicotine exposure (which activates nAChRs), BDNF in the hippocampus is upregulated (114, 115). Conversely, after loss of basal forebrain cholinergic neurons, hippocampal BDNF subsequently decreases (116). These data have been interpreted as a direct effect of ACh on neurons and this view is supported by the finding that in cultures of cortical neurons, α7 nAChR stimulation produces dose-dependent increases in BDNF. However, there is also evidence that microglia modulate BDNF release. For example, hippocampal microglial activation following LPS administration led to decreased BDNF in the CA1 region of the hippocampus (117). Two studies by Ruth Barrientos and colleagues show that hippocampal IL-1β, likely produced by microglia, regulates BDNF. In one study, social isolation stress lowered BDNF levels in the hippocampus of mice, but levels were restored when an IL-1 receptor antagonist was administered to the hippocampus (118). In a second study, the increase of hippocampal BDNF following context learning was blocked by

administering IL-1β to the hippocampus (119). High levels of IL-6 also suppress BDNF (120). There is at least some evidence that ACh modulation of inflammation can alter BDNF release. Vagal nerve stimulation, known to release ACh in the periphery and to be a catalyst for the cholinergic anti-inflammatory pathway, has been shown to upregulate both neurogenesis and BDNF in the hippocampus after 24 hours and 3 weeks of treatment (121). Though the mechanism of this effect is not yet known, it may be because vagal stimulation also enhances serotonin (*via* the raphe nucleus) and norepinephrine (*via* the locus ceruleus) in the CNS [see Biggio et al. (121)]. This increase in norepinephrine could lead to increased cholinergic signaling in the basal forebrain (122), contributing to increased hippocampal neurogenesis (123). Peripheral cholinergic stimulation (89) and vagal nerve stimulation (124) are also known to decrease neuroinflammation, which contributes to increased hippocampal neurogenesis (125).

Importantly, the effects of BDNF and activation of α7 nAChRs appear to be reciprocal. BDNF increases the density of α7 nAChRs on hippocampal neurons (126), and activation of α7 nAChRs leads to upregulated BDNF in the hippocampus (127). Together, these data suggest that the cholinergic system modulates BDNF and neural plasticity *via* both direct neuronal and indirect glial actions.

Neurogenesis

The dentate gyrus (DG) of the hippocampus is one of the most plastic regions in the mammalian brain because it is able to generate principal neurons that integrate into the pre-existing network throughout life. Moreover, basal forebrain cholinergic projections to the DG have been shown to facilitate neurogenesis (128–130). Enhanced adult hippocampal neurogenesis improves pattern separation ability, temporal separation of events in memory, forgetting, and cognitive flexibility [see recent review: Hvoslef-Eide and Oomen (131)]. Because these abilities rely on suppressing older memories and inputting new associations, they likely rely on cholinergic inputs that modify hippocampal neurogenesis.

Neural stem cells in the hippocampus express ACh receptors, including mAChRs and α7 nAChRs (130), providing a possible mechanism by which the cholinergic system influences neurogenesis. In general, *in vivo* and *in vitro* studies show that cholinergic receptor stimulation increases neural stem cell proliferation (132). Activation of the α7 nAChR *via* increased ACh levels has been shown to enhance new neuron survival, but not differentiation or proliferation [see Kita et al. (133) and Narla et al. (134) for reviews]. These manipulations may be influencing neuronal progenitors through ACh receptors, but they also may be impacting neuronal proliferation and survival by acting on microglia. However, this possibility has not been addressed directly.

Cholinergic stimulation *via* increased ACh levels also promotes hippocampal neurogenesis, and decreased ACh levels impair it (123, 135). There is some evidence that a high-choline diet in adulthood, leading to increased ACh synthesis (136), increases proliferation and/or survival of hippocampal neurons (137). If choline-induced proliferation occurred, then it was likely due to a different mechanism than the α7 nAChR because of previous research implicating this receptor in neuronal survival but not proliferation [see Kita et al. (133) and Narla et al. (134) for reviews]. AChE inhibitors also upregulate proliferation of cells in the DG (129, 130, 132) and exercise-induced proliferation of aged neural stem cells is prevented by lesions of the septal cholinergic system (132, 138). These findings indicate that neural stem cells respond to cholinergic stimulation even in aged animals.

As was true for memory function, the immune system is needed for neurogenesis, but overactivation leads to a decrease in neurogenesis. Mice lacking T- and B-cells have impaired hippocampal neurogenesis, which is rescued by reintroducing T-cells in the periphery (139). Because some of the reintroduced T-cells likely had the ability to make and release ACh, it is possible that the restoration of neurogenesis was *via* increases in ACh. As with LTP, DG neurogenesis requires some microglia (140). However, microglial overactivation, for example following stress, infection, or disease, appears to compromise neurogenesis, which may contribute to the memory impairments seen in these conditions. When the immune system is activated by stress, minocycline decreases microglial activation and rescues adult hippocampal neurogenesis in mice (141). Minocycline also rescues neurogenesis in AD-model and schizophrenia-model mice that have been exposed to LPS (142–144). IL-6 and TNF-α have been shown to decrease hippocampal neurogenesis in adulthood when overexpressed (125), providing further evidence that an increase in neuroinflammation leads to a decrease in neurogenesis.

In addition to impairing neurogenesis, inflammatory cytokine IL-6 is neurotoxic. In a study analyzing amyotrophic lateral sclerosis (ALS) blood cells, which normally secrete TNF-α and IL-6, researchers found that these cells were toxic to rat neurons *in vitro* (145). Adding an anti-IL-6 antibody blunted the toxicity, and anti-TNF-α and anti-IL-1β antibodies did so to a lesser extent. This study in particular is critical in parsing out the effects of the often-grouped-together cytokines (TNF-α, IL-1β, and IL-6): IL-6 is shown to play the largest role of the three in neurotoxicity. Additionally, overexpression of IL-6 led to more neurodegeneration by several metrics (146). Inflammatory cytokines released by microglia lead to impaired neurogenesis and increased neurodegeneration. There have not been specific studies elucidating the role of microglial α7 nAChRs in neurogenesis, but it is possible that their activation leads to fewer inflammatory cytokines, leading to increased neurogenesis and cell survival. Together, these data suggest that cholinergic stimulation of the DG may modify hippocampal neurogenesis directly *via* stimulation of ACh receptors on DG stem cells or indirectly *via* stimulation of microglia and the subsequent inhibition of IL-6 and TNF-α.

CLINICAL APPLICATIONS

Inflammation and the central release of inflammatory cytokines have been proposed as a mechanism underlying cognitive decline or dysfunction in mouse models of AD [reviewed in Mosher and Wyss-Coray (147)], stress (148), cancer-treatment (e.g., irradiation and chemotherapy) (149, 150), multiple sclerosis (151), and obesity (152). The degeneration of the basal forebrain cholinergic system is a factor in many forms of dementia: not only in AD, but also in Parkinson's disease, Down syndrome, ALS, and supranuclear palsy [see Ferreira-Vieira et al. (60)]. One likely possibility that is sometimes overlooked is that the loss of cholinergic input

in these disorders may unmask inflammation in the hippocampus, leading to impaired cognition.

Parkinson's Disease

Parkinson's disease, while characterized by motor deficits, produces impairments in working memory, attention, and a two to six times higher risk of dementia (153, 154). Mouse models show decreased cortical ACh and impaired performance in hippocampal tasks such as the Y-maze, novel-object recognition, object-place recognition, and operant reversal learning (155). Activation of nAChRs, including the α7 nAChR, is neuroprotective in animal models of Parkinson's disease [reviewed in Quik et al. (156)]. In Parkinson's patients, lower cholinergic activity, lower basal forebrain volume, and cholinergic denervation in the thalamus and cortex are all associated with impaired cognition (38, 157–159). Based on this evidence, the cholinergic deficits underlying Parkinson's disease may be causally linked to the memory deficits.

While Parkinson's disease causes a loss of dopaminergic neurons, it also impacts the cholinergic system and produces neuroinflammation. For example, in a mouse model of Parkinson's, microglia and astrocytes in the substantia nigra are increased in number and show more activation-associated morphology compared to controls (24). Microglial number, as well as astrocyte number and activation, were decreased following application of a nAChR agonist. Alpha7 nAChR activation prevents dopaminergic cell death by inhibiting the activation of both astrocytes and microglia in the substantia nigra (24). These findings suggest that Parkinson's is inherently inflammatory, and this inflammation is decreased by activating nAChRs. This inflammatory profile may be either the cause or a result of dopaminergic cell death. If nAChR activation, and hence activation of the cholinergic anti-inflammatory pathway in the brain, prevents dopaminergic cell death, it is possible that the inflammation itself has a role in neurodegeneration in Parkinson's. Therefore, blunting neuroinflammation by activating α7 nAChRs might be a promising therapy to prevent neuron loss in these patients. Though this study examines the substantia nigra, it is possible that inflammation is also occurring in the hippocampus, which would then contribute to loss of cognitive function seen in Parkinson's.

Cholinergic drugs have, in fact, been used to treat Parkinson's-associated cognitive deficits with some success [see Pagano et al. (160) for a meta-analysis]. Interestingly, the prevalence of Parkinson's disease is decreased in smokers [see Quik et al. (161)] suggesting that nicotine may be neuroprotective, though the target of nicotine action for this beneficial effect is not yet known [see Piao et al. (162)]. It has been suggested that neuroinflammation may not just be the result of the cell loss seen in Parkinson's disease, but that noradrenergic and cholinergic hypofunction may contribute to dysregulation of neuron-glia interactions, leading to inflammation and, eventually, neurodegeneration (163). Thus, a better understanding of the role of cholinergic systems in modulating communication between glia and neurons may lead to the development of cholinergic drugs that could be promising for prevention or treatment of Parkinson's disease.

Alzheimer's Disease

Cholinergic neurons in the basal forebrain selectively degenerate in AD (36, 164, 165), and these cells are also the first neurons effected in early AD [see the following reviews (40, 166, 167)]. Impaired cognitive function in AD is associated with increased neurofibrillary tangle density in the basal forebrain (168). As well, postmortem brains of AD patients have lower levels of ChAT and AChE, regardless of age (169). These findings led to the "cholinergic hypothesis" for AD and to the treatment of AD patients with AChE inhibitors. The widely held view is that this drug treatment helps to negate the loss of cholinergic neurons by increasing ACh postsynaptic action. In contrast, not much work has been devoted to the effects of these drugs on glia in AD patients (170).

AD is also characterized by a heightened profile of neuroinflammation [see Calsolaro and Edison (171)]. AD patients have more TNF-α and IL-6 in both serum and brain, indicating an inflammatory phenotype (172, 173), and higher levels of TNF-α are highly correlated with rapid cognitive decline (174). Post-mortem AD brains also have more activated microglia and astrocytes (175). Microglia in particular have been implicated in the excessive neuronal loss seen in AD (176) and overactivation of microglia is seen relatively early in the progression of the disease (177). Thus, AD neuropathology is characterized not only by a loss of cholinergic neurons but also by increased neuro-inflammation. Interestingly, peripheral inflammation has been linked with early-onset AD more so than with late-onset type AD (178), suggesting that inflammation may be more critical feature in the development or progression of the familial form of the disease.

As in Parkinson's disease, it is still not clear exactly how the innate immune system is involved in AD, specifically whether it is a cause or effect of pathology. Some studies show that neuroinflammation, though contributing to an AD phenotype, is initiated by amyloid-β and thus occurs as a result of AD neuropathogenesis. For example, microglial TNF-α was shown to catalyze a cascade of cellular events *in vitro* that characterize AD, including cell toxicity (179). Amyloid-β peptides also appear to activate microglia, which initiate the toxic "cell cycle events" *via* TNF-α. In contrast, TNF-α knockout mice do not show these toxic "cell cycle events"—powerfully implicating this cytokine in the neuronal death seen in AD. Because there are α7 nAChRs on microglia, perhaps activation of these receptors could have a hand in preventing AD pathology.

It is worth mentioning that the interaction between the cholinergic and neuroimmune systems differs in AD versus normal aging [see Schliebs and Arendt (180)]. Specifically, cholinergic cell *loss* is seen in AD, but cholinergic *dysfunction* (in the form of synaptic losses and other modifications) is seen in normal aging (36, 181–183).

However, the link between TNF-α, IL-1β, and IL-6 and AD is not completely clear. Expression of these cytokines in blood serum is increased in early-onset AD patients, but not late-onset AD patients (178). TNF-α expression decreases the presence of amyloid-β plaques in the mouse hippocampus (184), indicating that these cytokines may actually decrease AD pathology. Clearly, more research is needed into the complicated role of inflammatory cytokines in AD patients.

Cholinomimetic drugs, especially AChE inhibitors, have been the first line of defense in AD treatment for many years, and as mentioned above, their benefits are thought to be due to a synaptic increase in ACh or direct stimulation of ACh receptors. However, some of the benefits of these drugs in AD patients may be due to decreases in inflammation in addition to their synaptic actions. Levels of IL-6 are higher in the brains of AD patients—however, this level decreases dose-dependently based on how many months the patients have taken AChE inhibitors (185). Possibly, by acting on microglial α7 nAChRs, cholinergic drugs may decrease the release of inflammatory cytokines and slow neurodegeneration.

Neuroinflammation following neurodegeneration and other neuropathologies (plaques and tangles) likely contributes to clinical impairments in memory (and perhaps other symptoms as well) in both Parkinson's disease and AD, and speeds progression of these diseases. However, it is also possible that breakdown in the innate immune system, leading to altered glial–neuron responses, contributes to the onset of AD and Parkinson's in different ways. Dursun et al. (178) compared peripheral cytokine levels in early-onset AD, late-onset AD, mild cognitive impairment, and Parkinson's disease. Though early-onset AD patients displayed increased levels of inflammatory cytokines, Parkinson's disease patients exhibited a different profile: IL-1β was increased, but IL-6 was significantly decreased. The interaction between these two cytokines appears to be different in AD and Parkinson's disease, suggesting that the glial contributions to these diseases may be different.

CONCLUSION

Acetylcholine has multiple mechanisms by which it can modulate hippocampal memory: ACh binds directly to neuronal pre- and postsynaptic receptors, initiating downstream neuronal actions, and to receptors on astrocytes and microglia to decrease pro-inflammatory cytokines and increase the release of growth factors like BDNF. ACh can also act *via* the peripheral or central anti-inflammatory pathways by suppressing overactivation of peripheral macrophages or central microglia to indirectly aid memory. Not only does evidence point to multiple effects of ACh on memory processes, there are also multiple effects of ACh on neuroplasticity—specifically, alteration of spine density, synaptic strength, BDNF, and hippocampal neurogenesis. These findings suggest new ways of preventing age-related memory decline and perhaps delaying or preventing the cognitive impairments accompanying neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

SVM wrote this manuscript with significant contribution in ideas, structure, and editing from CLW.

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Forebrain Cholinergic Dysfunction and Systemic and Brain Inflammation in Murine Sepsis Survivors

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Sepsis, a complex disorder characterized by immune, metabolic, and neurological dysregulation, is the number one killer in the intensive care unit. Mortality remains alarmingly high even in among *sepsis survivors* discharged from the hospital. There is no clear strategy for managing this lethal chronic sepsis illness, which is associated with severe functional disabilities and cognitive deterioration. Providing insight into the underlying pathophysiology is desperately needed to direct new therapeutic approaches. Previous studies have shown that brain cholinergic signaling importantly regulates cognition and inflammation. Here, we studied the relationship between peripheral immunometabolic alterations and brain cholinergic and inflammatory states in mouse survivors of cecal ligation and puncture (CLP)-induced sepsis. Within 6 days, CLP resulted in 50% mortality vs. 100% survival in sham-operated controls. As compared to sham controls, sepsis survivors had significantly lower body weight, higher serum TNF, interleukin (IL)-1β, IL-6, CXCL1, IL-10, and HMGB1 levels, a lower TNF response to LPS challenge, and lower serum insulin, leptin, and plasminogen activator inhibitor-1 levels on day 14. In the basal forebrain of mouse sepsis survivors, the number of cholinergic [choline acetyltransferase (ChAT)-positive] neurons was significantly reduced. In the hippocampus and the cortex of mouse sepsis survivors, the activity of acetylcholinesterase (AChE), the enzyme that degrades acetylcholine, as well as the expression of its encoding gene were significantly increased. In addition, the expression of the gene encoding the M1 muscarinic acetylcholine receptor was decreased in the hippocampus. In parallel with these forebrain cholinergic alterations, microglial activation (in the cortex) and increased *Il1b* and *Il6* gene expression (in the cortex), and *Il1b* gene expression (in the hippocampus) were observed in mouse sepsis survivors. Furthermore, microglial activation

was linked to decreased cortical ChAT protein expression and increased AChE activity. These results reinforce the notion of *persistent inflammation-immunosuppression and catabolic syndrome* in sepsis survivors and characterize a previously unrecognized relationship between forebrain cholinergic dysfunction and neuroinflammation in sepsis survivors. This insight is of interest for new therapeutic approaches that focus on brain cholinergic signaling for patients with chronic sepsis illness, a problem with no specific treatment.

Keywords: sepsis, sepsis survival, cytokines, inflammation, brain cholinergic system, neuroinflammation

INTRODUCTION

Sepsis is a clinical syndrome manifested by a spectrum of immune, metabolic, and neurological derangements. Although sepsis remains the most frequent cause of death in intensive care units, advances in critical care medicine have substantially reduced sepsis mortality during hospitalization (1, 2). However, sepsis continues to kill for a long time after patients are discharged from the hospital (3–6). Many *sepsis survivors* are unable to return to independent living and develop chronic illness, characterized by severe functional disabilities, which ultimately result in death (4–8).

The brain is severely affected in sepsis. In acute settings a frequent event is sepsis-associated encephalopathy, which ranges from confusion and delirium to coma (9). Among other derangements, brain neurotransmission dysregulation, microglial activation, and proinflammatory signaling have been described within the spectrum of this characteristic encephalopathy (9–11). This neurological complication is an independent predictor of sepsis mortality (9). Persistent long-term cognitive impairment has been also reported in survivors of sepsis and other critical illnesses (3, 4, 12). This cognitive impairment can be as severe as the one observed in moderate traumatic brain injury and mild Alzheimer disease (13). Pathophysiological events in the brain underlying cognitive impairment in the context of other functional disabilities associated with metabolic and immune dysfunction remain poorly understood. Recently, the condition of *persistent inflammation-immunosuppression and catabolic syndrome* was described in sepsis survivors, who develop debilitating and lethal chronic illness (14, 15). Peripheral inflammation and increased circulating cytokine levels have been linked to brain proinflammatory signaling and altered brain function (16, 17). In a murine model, systemic inflammation during endotoxemia was indicated as a trigger of profound glial activation and apoptotic neuronal death in the hippocampus (18). Increased brain cytokine and chemokine transcription with microglial activation and decreased neuronal cell density in the cortex and hippocampus of endotoxemic rats was also reported (19). Cecal ligation and puncture (CLP)-induced sepsis in mice results in impaired learning and memory, along with chronic loss of dendrites in the hippocampus (20, 21). In murine sepsis survivors, proinflammatory signaling induced by HMGB1 mediates cognitive deterioration (21). A recent study also reported long-term brain alterations in mouse sepsis survivors, including forms of cognitive deficit and neuroinflammation (22).

Cognition, including attention, learning, and memory, is regulated by brain cholinergic signaling. Cholinergic neurons in the basal forebrain constitute a major neuromodulatory system with a critical role in this regulation (23, 24). Neurodegeneration of basal forebrain cholinergic neurons is implicated in cognitive impairment in Alzheimer's disease (23). These neurons also regulate neuroplasticity allowing functional recovery following brain injury (25). Recent findings have also characterized a role for brain cholinergic signaling in controlling peripheral immune responses and inflammation (26–32). Despite the important involvement of the brain cholinergic system in cognition and the neural control of inflammation, the understanding of its function in the context of sepsis is limited. We recently reported altered gene expression of brain cholinergic system markers in parallel with increased circulating cytokine levels and inflammation in the brain (neuroinflammation) of endotoxemic mice (33). Reduced cholinergic innervations of cortical areas and neuronal loss in the hippocampus and long-term cognitive impairment, similar to that observed in Alzheimer's disease, was reported in rats that survived endotoxemia (34). Here, we studied the relationship between peripheral immune and metabolic alterations and forebrain cholinergic signaling and neuroinflammation in murine survivors of CLP-induced sepsis.

MATERIALS AND METHODS

Animals

Male BALB/c mice (24–28 g, Taconic) were used in experiments. Animals were allowed to acclimate for at least 2 weeks prior to initiating the experiment. All animals were housed in standard conditions (room temperature 22°C with a 12 h light–dark cycle) with access to regular chow and water. All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the Feinstein Institute for Medical Research, Northwell Health, Manhasset, NY, USA.

Cecal Ligation and Puncture

A standardized model of CLP-induced severe polymicrobial sepsis was used, as previously described (31, 35, 36). Mice were anesthetized using ketamine 100 mg/kg and xylazine 8 mg/kg, administered intramuscularly. Abdominal access was gained *via* a midline incision. The cecum was isolated and ligated with a 6-0

silk ligature below the ileocecal valve and then punctured once with a 22 G needle. Stool (approximately 1 mm) was extruded from the hole, and the cecum was placed back into the abdominal cavity. The abdomen was closed with two layers of 6-0 Ethilon sutures. An antibiotic, Primaxin (Imipenem-Cilastatin, 0.5 mg/kg, subcutaneously, in a total volume of 0.5 ml/mouse) was administered immediately after CLP as part of the resuscitation fluid. Mice were monitored for survival and sepsis-associated clinical signs twice daily for the first 7 days, and then daily for the remaining of 14 days. Sham-operated animals had the cecum isolated and then returned to the peritoneal cavity without being ligated or punctured. Sham animals also received antibiotics and resuscitative fluid as described above.

Brain Tissue Preparation

After collecting blood for cytokine analysis, brains were isolated on ice. For brain immunostaining, brain isolation was preceded by transcardial perfusion with saline and 4% paraformaldehyde. For acetylcholinesterase (AChE) activity determination and gene expression by qPCR, the cerebral cortices and the hippocampi were dissected on ice using a binocular dissection microscope. Brain tissue was snap frozen on dry ice and transferred to storage at −80°C.

Serum Cytokine and Metabolic Molecule Determination

Blood was collected immediately after euthanasia by cardiac puncture. To obtain serum samples, blood was allowed to clot for 1.5 h and centrifuged at 5,000 rpm (1,500 *g*) for 10 min. Supernatants (sera) were collected and stored at −20°C until cytokine analyses. Interleukin (IL)-6, IL-1β, chemokine (C-X-C motif) ligand (CXCL1), IL-12p70, IL-5, IL-10, and TNF were determined by using the V-PLEX proinflammatory panel 1 mouse kit (Meso Scale Discovery, Gaithersburg, MD, USA), according to manufacturer's recommendations. HMGB1 was determined by western blot as previously described by Yang et al. (37). Insulin, leptin, plasminogen activator inhibitor-1 (PAI-1) (total), and resistin were measured by using milliplex map mouse adipokine magnetic bead panel—endocrine multiplex assay (Millipore, USA) according to manufacturer's recommendations.

Brain Sample Isolation and qPCR Analysis

Fresh brains were collected in RNA later (Ambion®) 2 weeks following CLP. RNA isolation from cortex and hippocampus was performed using RNeasy Plus mini kit (Qiagen, Germantown, MD, USA) after tissue homogenization with the Bullet Blender Homogenizer (Next Advance, Averill Park, NY, USA) and the recommended bead lysis kit. Because of limited RNA levels found in the hippocampus, three tissue samples were combined from the same treatment groups before RNA extraction. RNA concentrations were measured using NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Specific primers for mouse *Il1b*, *Il6*, *Ache*, *Chat, Chrm1*, and *Gapdh* (internal reference gene) were designed using the Universal Probe Library Assay Design Center (Roche Applied Sciences, Indianapolis, IN, USA) with the assigned probes as listed in **Table 1**. RNA (100 ng/reaction) was amplified using a Eurogentec reverse-transcriptase qPCR master mix (AnaSpec, EGT Corporate Headquarters, Fremont, CA, USA) on a LightCycler 480 (F. Hoffmann-La Roche Ltd., Basel, Switzerland), and the data were analyzed by using the Roche LightCycler 480 SW 1.5 software. Relative changes in mRNA expression were calculated as fold-changes (normalized using *Gapdh*) by using the comparative Ct (ΔΔCt) method (38).

TABLE 1 | Probes used in qPCR analyses.

Primer	Probe
Il1b	Left AGTTG ACGGACCCCAAAAG Right AGCTG GATGCTCTCATCAGG
Il6	Left GCTACCAAACTGGATATAAT CAGGA Right CCAGGTAGCTATGGT ACTCCAGAA
Ache	Left TTAGGGCTGGGATATAATACGAC Right GCCCCTAGTGGGAGGAAGT
Chat	Left AAGCTTCCACGCCACTTTC Right AGAGCCTCCGACGAA GTTG
Chrm1	Left GGTCCCAGGAGACACTGC Right TCAGAGTAAGGGCATCACCA
Gapdh	Left GAGCCCGCAGCCTCCCGCTT Right CCCGCGGCCATCACGCCACAG

Acetylcholinestrase (AChE) Activity Assay

Brain tissue homogenates from cortex and hippocampus were prepared in a 20 mM Tris-HCl buffer (pH7.3), containing 10 mM $MgCl_2$, 50 mM NaCl, a protease inhibitor and zirconium beads using a bullet-blender homogenizer (Next Advance, Troy, NY, USA), according to the manufacturer's recommendations. Homogenates were centrifuged (24,500 *g*) and the pellets were resuspended in equal volumes of 0.1 M $NaHPO_4$ buffer (pH 7.4–8.0) and 0.1% Triton-X-100. Following centrifugation (24,500 *g*) the resultant supernatants (containing membrane bound cholinesterase) were used an AChE activity assay based on the widely used Ellman's procedure (39) and its modification (40). A butyrylcholinesterase inhibitor was added to the reaction mix. The reaction was initiated by adding acetyl thiocholine substrate, a thiolester. The amount of thiocholine formed reflects AChE activity. The color of the reaction mix was read at 412 nm. Calculations were performed using molar absorptivity (ε) for thionitrobenzoate at 412 nm. The results are expressed as millimoles of thiocholine released per minute at 25°C per 1 ml of lysate per 1 mg of protein.

Immunohistochemical Staining

Following euthanasia by CO_2 asphyxiation, animals were perfused transcardially with saline, and then 4% paraformaldehyde. Whole brain tissue was fixed in 4% paraformaldehyde for 24 h, and then processed, paraffin embedded, and sectioned sagittally at 6 μm thickness using a microtome (Leica Biosystems). The Paxinos and Franklin mouse brain atlas (41) was used to establish laterality (on the coronal plane) for serial sections to include areas of interest. For immunofluoresence, following deparaffinization and antigen retrieval, sections were incubated

for 2 h at room temperature in TBS + 1% Triton-X + 10% donkey serum. Sections were incubated for 24 h at 4°C with primary antibodies, followed by 2 h incubation at room temperature with the appropriate secondary antibody with DAPI. All images were captured on a confocal microscope (Olympus Fluoview 300 Confocal Microscope). The following primary antibodies were used: anticholine acetyltransferase (anti-ChAT) antibody [Millipore (1:100) Billerica, MA, USA] and Iba-1 [Wako (1:400), Richmond, VA, USA]. The secondary antibodies which were used include Cy3 donkey antigoat IgG Cy3 and donkey antirabbit IgG Alexa Fluor 488 (Jackson Immunoresearch, 1:125, West Grove, PA, USA). For negative control, sections were incubated with TBS + 1% Triton-X + 10% donkey serum for 24 h (no primary antibody added) followed by 2 h incubation with the appropriate secondary antibody with DAPI.

Immunostaining Analysis

Digital images were obtained using Confocal software and were exported to Image J. Excitation and acquisition parameters were adjusted to fully eliminate pixel saturation and all images were collected under identical settings. Cholinergic (ChAT-positive neurons) were identified in the basal forebrain on the sagittal plane by referring to Allen Institute Allen Brain Atlas (http://mouse.brain-map.org). Cell counting (of ChAT positive neurons) was performed on four sections per animal (750 × 750 µm each) and four animals per group by an observer blinded to the experimental group.

Cortical Neuron Cultures and LPS Microglial Activation

Cortical neuronal cultures were prepared from BALB/c mouse pups, as previously described (42) with some modifications. Briefly, postnatal day 0 newborn pup brains were harvested for neuronal cultures. Cortical gray matter was dissected, plated separately in six-well plates at 1×10^6 per well (previously coated with laminin and poly-L-Ornithine, Sigma, St. Louis, MO, USA). Cultures were fed with neurobasal medium with B-27 supplements (Thermofisher, Waltham, MA, USA) and incubated at 37°C with 5% CO_2. Seven-day-old neuronal cultures were treated for 16 h with 0.5 ml per well of microglial conditioned medium. Microglial conditioned media was obtained by incubating mouse microglia BV2 cells (ATCC CRL-2469) with LPS (10 µg/ml) in a humidified incubator (5% CO_2, 37°C and 95% air) for 24 h.

Choline Acetyl Transferase Protein Determination

Protein lysate was extracted from mouse primary cortical neuronal cell culture. Protein concentration was estimated using the Modified Lowry Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). Standard SDS-PAGE techniques were followed. After electrophoresis, proteins were transferred to a PVDF membrane using a Wet/Tank Blotting System (Bio-Rad, Hercules, CA, USA). Membranes were briefly washed, incubated with specific primary antibodies: goat anticholine acetyl transferase (ChAT; 1:1,000 Millipore, Billerica, MA, USA) or rabbit antiactin (1:1,000; Abcam, Cambridge, MA, USA) in 5% BSA with PBST overnight. After washing, the membranes were incubated with anti-goat HRP and anti-rabbit secondary antibodies (1:10,000) for 60 min, washed, processed using Amersham ECL detection systems (GE Healthcare, Piscataway, NJ, USA) and exposed to 8 × 10 Fuji X-Ray Film. Lysate from rat forebrain was used as positive control. Densitometric analysis was performed using Image J software.

Statistical Analysis

All statistical tests were performed with Graph Pad Prism 6 software. Values are presented as mean ± SEM. Statistical analysis of mean differences between groups was performed by unpaired two-tailed Student's *t*-test. All *P*-values and *n* values are indicated in figure legends and in the text. *P*-values ≤0.05 were considered significant.

RESULTS

Immune and Metabolic States Are Altered in Murine Sepsis Survivors

First, we examined indices of the peripheral immune state and some metabolic markers in sepsis survivors. Two sets of mice with equal body weights were subjected to either CLP or sham surgery and survival was monitored for 14 days. A mortality rate of 50% was observed by day 6 with no further mortality following CLP ($n = 9$ per group) (**Figure 1A**). No mortality was observed in mice subjected to sham surgeries. The body weight of sepsis survivors was significantly lower when compared with sham controls 14 days postsurgery ($n = 9$ per group) (**Figure 1B**). Peripheral inflammation was examined in sepsis survivors and sham controls by determining serum cytokines levels. Fourteen days after the onset of disease, serum levels of TNF ($P = 0.0004$), IL-1β ($P = 0.0002$), IL-6 ($P < 0.0001$), CXCL1 ($P = 0.0091$), IL-10 ($P = 0.0002$), and HMGB1 ($P = 0.0001$) were significantly higher in CLP mice when compared with sham controls ($n = 8$–9 per group) (**Figure 2A**). No difference in serum IL-12p70 between the groups was observed (**Figure 2A**). In addition, very low IL-5

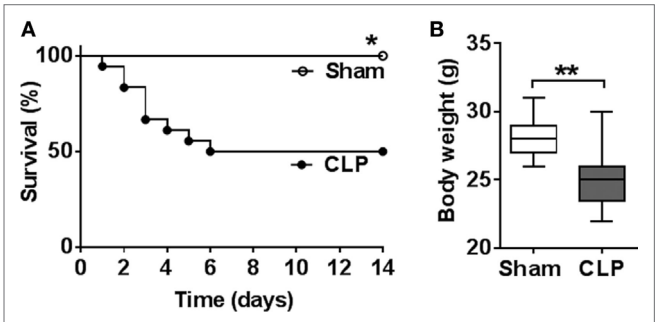

FIGURE 1 | Sepsis mortality following cecal ligation and puncture (CLP) surgery and weight loss in sepsis survivors. **(A)** CLP causes mortality rate of 50% by day 6 and no later mortality until day 14 following surgery. *$P = 0.0142$, Log-rank test, $n = 9$ per group. **(B)** Fourteen days following CLP, mice exhibit lower body weight as compared to sham-operated controls. ***$P = 0.0052$, Student's *t*-test, $n = 9$ per group.

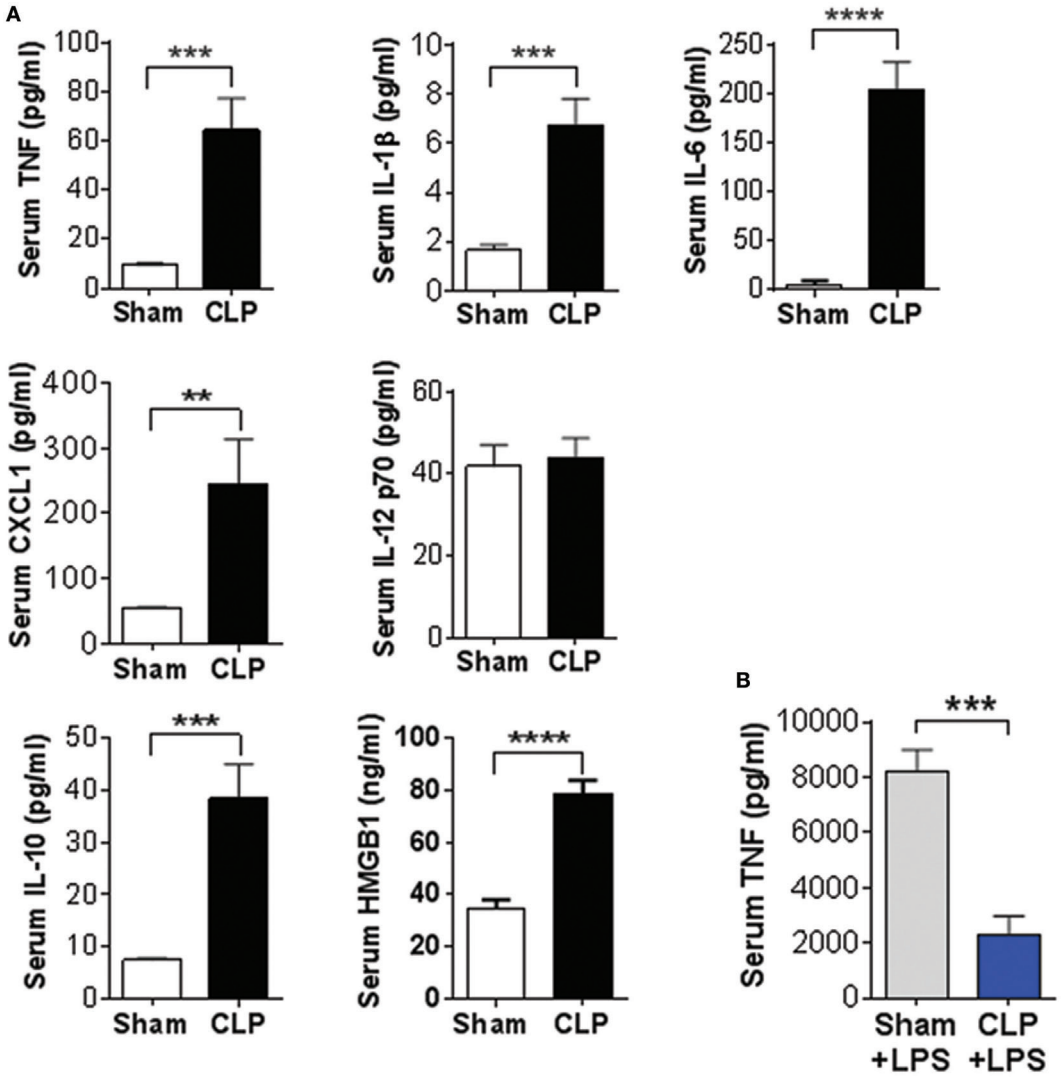

FIGURE 2 | Cecal ligation and puncture (CLP)-sepsis alters circulating cytokine levels and suppresses LPS-induced immune responsiveness. **(A)** Increased serum cytokine levels in mouse sepsis survivors 14 days after CLP or sham surgery. $^{**}P = 0.0092$, $^{***}P = 0.0004$ (TNF), $^{***}P = 0.0002$ (IL-1b, IL-10), $^{****}P = 0.0001$, Student's *t*-test, $n = 7$–9 per group. **(B)** Sepsis survivors exhibit reduced LPS-induced serum TNF levels during endotoxemia when compared to sham controls. $^{***}P = 0.001$, Student's *t*-test, $n = 8$–9 per group.

levels were determined in sham-operated controls and these levels were even lower in CLP mice (1.64 ± 0.19 vs. 0.87 ± 0.08 pg/ml, $P = 0.027$). To further characterize the impact of CLP on innate immune responsiveness, LPS (1 mg/kg, i.p.) was administered to sepsis survivors and sham mice 14 days after CLP- or sham-surgery and animals were euthanized 1.5 h later. As shown in **Figure 2B** endotoxin-induced serum TNF levels in CLP mice were fourfold lower ($P = 0.001$) than levels found in the sham controls ($n = 8$–9 per group). In addition to the inflammatory state, circulating levels of metabolic indices were altered in sepsis survivors. Circulating insulin ($P = 0.0023$), leptin ($P = 0.0157$), and PAI-1 ($P = 0.0109$) levels were significantly lower in CLP-survivors when compared to sham controls ($n = 8$–9 per group) (**Figure 3**). No difference in serum resistin levels between the groups was observed (**Figure 3**). Together these results indicate the presence of peripheral immune and metabolic dysregulation in mouse sepsis survivors.

Forebrain Cholinergic Transmission Is Altered in Mice Sepsis Survivors

To study the impact of CLP on the forebrain cholinergic transmission in sepsis survivors, we examined cholinergic neuronal bodies (somata) in the basal forebrain and molecular cholinergic components in two major projection areas of these neurons—the cortex and the hippocampus. ChAT, the enzyme responsible for the biosynthesis of acetylcholine, is a widely used marker for identifying cholinergic neurons. ChAT immunostaining revealed that the number of ChAT-positive neurons in the basal forebrain was significantly reduced in sepsis survivors

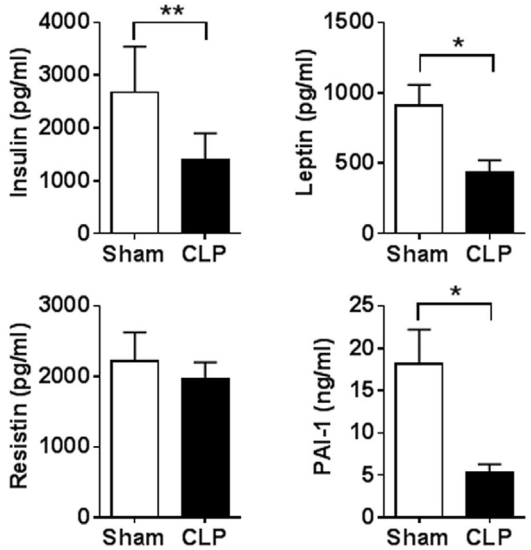

FIGURE 3 | Cecal ligation and puncture (CLP)-sepsis alters metabolic markers. Serum levels of insulin, leptin, and plasminogen activator inhibitor-1 (PAI-1) were decreased in mouse sepsis survivors. *P = 0.0157 (leptin), *P = 0.0109 (PAI-1), **P = 0.0023, Student's t-test, n = 8–9 per group.

compared to sham controls (n = 5 per group) (**Figures 4A,B**). ChAT colocalization with DAPI (Figure S1 in Supplementary Material) was used to precisely quantify ChAT-positive somata. Acetylcholine, released from cholinergic neurons, is rapidly degraded by AChE in the synaptic cleft between cholinergic and cholinoceptive neurons (23). AChE activity in the hippocampus was significantly increased among sepsis survivors (n = 8 per group) (**Figure 4C**). In addition, upregulation of *Ache* mRNA expression in the cortex was observed (n = 8 per group) (**Figure 4D**). Acetylcholine receptors on postsynaptic cholinoceptive neurons mediate cholinergic neurotransmission in the synaptic cleft. The M1 mAChR is predominantly postsynaptic, with a major role in processing cholinergic transmission in the cortex and hippocampus (43, 44). The expression of the gene encoding the M1 mAChR receptor, *Chrm1* was decreased in the hippocampus of mouse sepsis survivors (n = 8 per group) (**Figure 4E**). Comparing *Ache* and *Chrm1* gene expression in the hippocampus and the cortex of sham control mice showed lower *Ache* and higher *Chrm1* mRNA expression in the hippocampus (Figure S2 in Supplementary Material). These observations highlight dysfunctional forebrain cholinergic signaling in the sepsis survivors.

Brain Inflammatory Indices Are Upregulated in Murine Sepsis Survivors

Next, we investigated inflammation in the brains of sepsis survivors by examining microglial activation and cytokine gene expression. Microglia, a major cell type with immune function in the brain mediates protective responses, but sustained microglial activation can have a deleterious impact on neuronal function (45, 46). Iba1 immunohistochemistry revealed morphological differences in microglia in the cortex between sepsis survivors and sham controls. Microglia in the cortex of sham operated controls exhibited typical ramified morphology, characterized by relatively small bodies and long, fine processes (n = 8 per group) (**Figure 5A**). In contrast, microglial hypertrophic cell bodies, and shortening and thickening of processes, indicative of activated microglia, were observed in the cortex of CLP mice (**Figure 5A**). In addition, a significant upregulation of *Iba1* gene expression was determined in the cortex of sepsis survivors when compared to sham controls (n = 8 per group) (**Figure 5B**). Microglial activation is associated with initiation of transcriptional pathways that lead to the release of cytokines and other inflammatory molecules (45). Accordingly, we found significantly upregulated *Il1b* gene expression in the cortex and hippocampus (n = 8 per group) (**Figure 5C**) and *Il6* gene expression in the cortex of mice following CLP (n = 8 per group) (**Figure 5D**). Astrocytes are another cell type in the brain that exhibit immune functions and astrogliosis is a distinct feature of neuroinflammation. Examination of astrocyte morphology using GFAP immunostaining revealed no gross differences between sepsis survivors and sham controls (data not shown). Collectively, these observations highlight the presence of sepsis-associated neuroinflammation with microgial activation and upregulated proinflammatory gene expression in the cortex and hippocampus of mice 14 days after CLP.

Microglial Activation Affects Cholinergic System Components in Primary Cortical Neurons

The relationship between microglial activation and the alterations in forebrain cholinergic signaling was further investigated by examining the impact of microglial activation on ChAT protein expression and AChE activity in primary cortical neurons. BV2 cells, a widely used murine microglial cell line (47), were incubated with 10 μg/ml LPS for 24 h and then the conditioned culture media was added to primary mouse cortical neurons for 16 h. In control experiments, basal conditioned BV2 culture media (in the absence of LPS) was added to primary mouse cortical neurons. Conditioned medium from LPS-activated microglial cells significantly decreased neuronal ChAT protein expression, as compared to control media treatment (microglial conditioned media in the absence of LPS) (P = 0.0343) (n = 3 per group) (**Figures 6A,B**). In addition, this treatment caused a significant increase in neuronal AChE activity in primary cortical neurons ($P < 0.0001$) (n = 3 per group) (**Figure 6C**). Together these observations suggest a causative role for activated microglia in altering cholinergic system components in cortical neurons.

DISCUSSION

Here, we show that mice surviving CLP-induced sepsis exhibit impaired immune and metabolic homeostasis, dysregulated forebrain cholinergic signaling, and neuroinflammation. In addition, our observations suggest that microglial activation, accompanying sepsis, can be linked to impaired brain cortical cholinergic signaling. These findings provide new insight into chronic sepsis illness and suggest potential therapeutic targets.

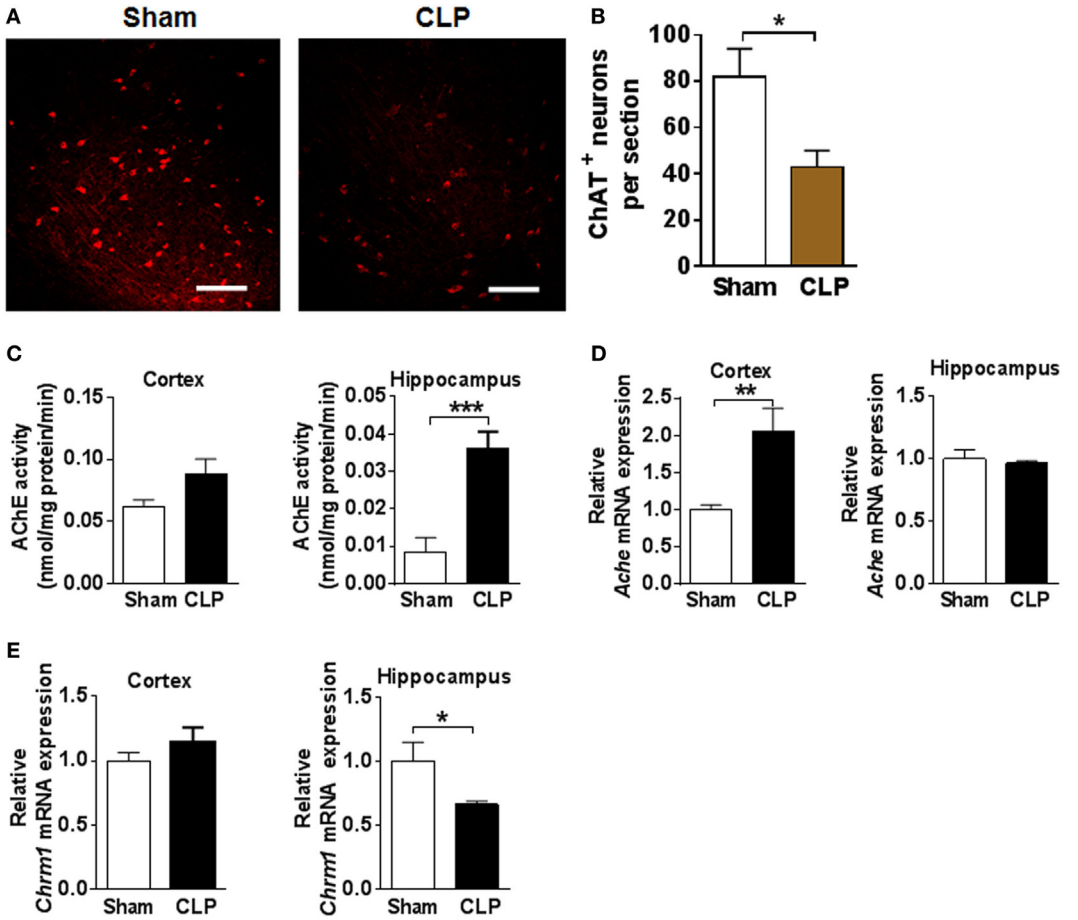

FIGURE 4 | Cecal ligation and puncture (CLP) has a significant impact on basal forebrain cholinergic balance in sepsis survivors. **(A)** Representative images of choline acetyltransferase (ChAT) immunostaining in the basal forebrain of sham and CLP mice (scale bar = 100 μm). **(B)** Quantitative analysis of ChAT immunostaining in the basal forebrain of sham and CLP mice. *P = 0.0229, Student's t-test, n = 5 per group. **(C)** Acetylcholinesterase (AChE) activity in cortex and hippocampus of sham and CLP mice. ***P = 0.0004, Student's t-test, n = 8 per group. **(D)** Relative *Ache* mRNA expression in cortex and hippocampus of sham and CLP mice. **P = 0.0052, Student's t-test, n = 8 per group. **(E)** Relative *Chrm1* mRNA expression in cortex and hippocampus of sham and CLP mice. *P = 0.0481, Student's t-test, n = 8 per group.

A growing number of patients surviving sepsis develop debilitating and lethal chronic illness. Recent data from two clinical trials revealed that the mortality of sepsis survivors (capable of independent living prior to severe sepsis) is approximately 30% within the first 6 months after the acute septic phase and the quality of life of one-third of the surviving patients is severely affected as indicated by their inability to return to independent living within 6 months (7). The increased risk of death among sepsis survivors persists for up to 5 years following the initial septic event, even after accounting for their underlying medical comorbidities (48). A recent study found that more than one fifth of patients surviving acute sepsis died within the first 2 years of causes not related to their health status before sepsis (5). Despite these staggering findings, there is no clear understanding of the pathophysiological events underlying the long-term sequelae of sepsis, including cognitive deterioration as a prerequisite for designing adequate treatments.

Here, we found increased serum IL-6, CXCL1, and HMGB1 levels in sepsis survivors 2 weeks post-CLP when compared to sham controls. Our current data describing dysregulated immune homeostasis in mouse sepsis survivors are in agreement with previous reports (49). In addition, higher serum IL-1β, and TNF and IL-10 levels were observed in the CLP survivors. The effect of CLP-sepsis on host innate immune responsiveness was investigated by administering endotoxin to sham mice and CLP survivors. CLP survivor mice showed significantly suppressed serum TNF levels following LPS challenge when compared to sham mice. These findings indicate the existence of a chronic inflammatory state in sepsis survivors, which coexists with suppressed innate immune function. This state, which we term *innate immune exhaustion* in sepsis survivors, may be a result of prolonged innate immune activation, which eventually leads to the inability of immune cells to adequately produce cytokines in response to immune challenges. Accordingly, early interventions directed toward neutralizing excessive innate immune activation may prevent or minimize immune suppression in sepsis survivors.

Metabolic dysregulation is a characteristic feature of sepsis pathobiology. Altered circulating levels of insulin, leptin,

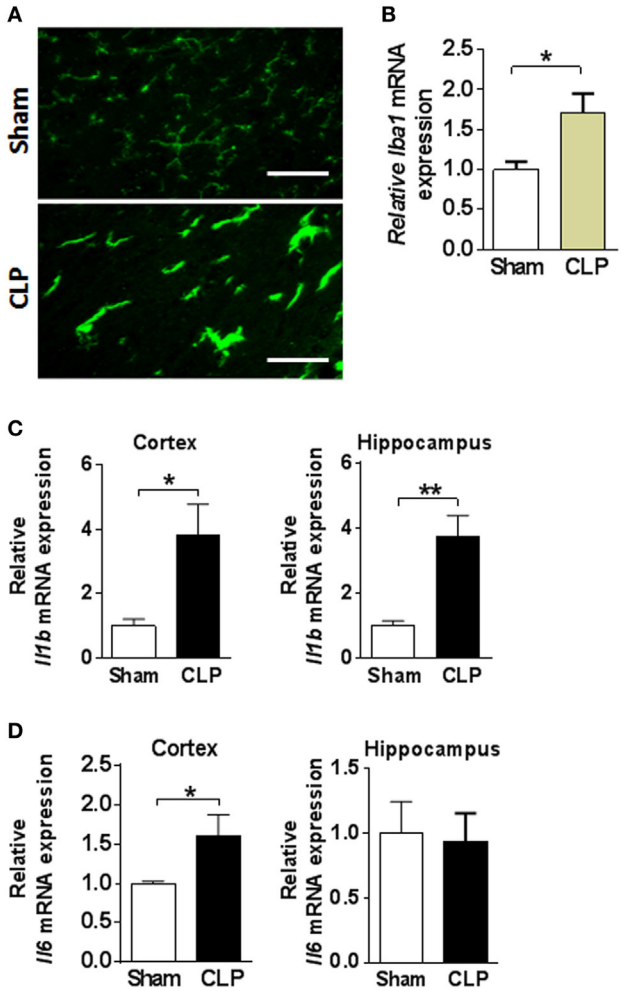

FIGURE 5 | Cecal ligation and puncture (CLP) results in neuroinflammation in mouse sepsis survivors. **(A)** Representative images of Iba1immunostaining in cortex of sham and CLP mice (scale bar = 50 μm). **(B)** *Iba1* mRNA expression in cortex and hippocampus of sham and CLP mice. *$P = 0.0139$, Student's *t*-test, $n = 8$ per group. **(C)** *Il1b* mRNA expression in cortex and hippocampus of sham and CLP mice. *$P = 0.0123$, **$P = 0.0011$, Student's *t*-test, $n = 8$ per group. **(D)** *Il6* mRNA expression *$P = 0.0455$, Student's *t*-test, $n = 8$ per group.

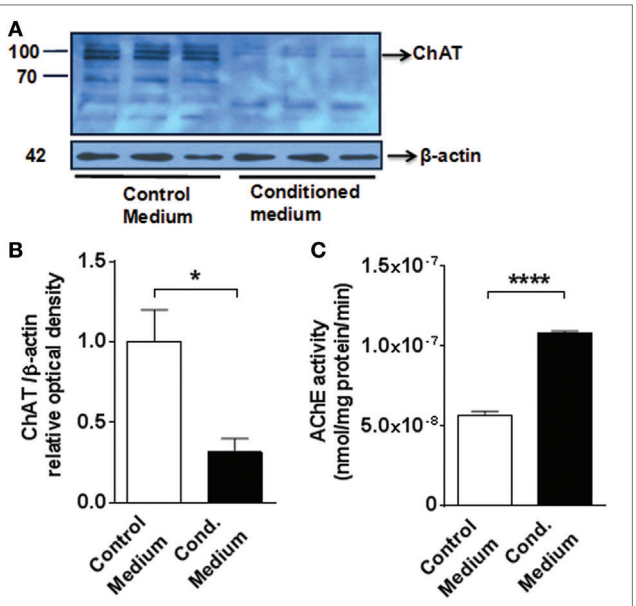

FIGURE 6 | Medium from LPS-activated microglial cells decreases choline acetyltransferase (ChAT) protein expression and increases acetylcholinesterase (AChE) activity in mouse primary cortical neurons **(A)** western blot (MW 83 kDa) of ChAT of 7-day-old primary neuronal culture exposed to control or conditioned media (obtained from LPS-activated microglial BV2 cells). See Section "Materials and Methods" for details. **(B)** Fold change of the western blot determined using Image J to measure band intensities of ChAT normalized to β-actin.*$P = 0.0343$, Student's *t*-test, $n = 3$ cultures per group, containing neurons obtained from five to six neonate brains each. **(C)** AChE activity in 7-day-old primary neuronal culture exposed to control or conditioned (Cond.) media ****$P < 0.0001$, Student's *t*-test, $n = 3$ cultures per group, containing neurons obtained from five to six neonate brains each. The Student's *t*-test was performed assuming normality and equal distribution of variance between the groups.

resistin, and PAI-1 have been previously indicated in acute settings of sepsis and these molecules have been implicated in sepsis pathogenesis (50, 51). However, no information is available about possible alterations in these metabolic molecules in sepsis survivors. Here, we found levels of insulin and leptin, a hormone and an adipokine associated with metabolic (predominantly anabolic) and immune functions (52) were lower in sepsis survivors. These results correlated with the lower body weight found in mouse sepsis survivors. We did not find a difference between the levels of the adipokine resistin in CLP survivors and sham controls. Levels of PAI-1, an important molecule in the processes of fibrinolysis and a validated fibrinolysis biomarker, were lower in sepsis survivors. PAI-1 is considered an independent risk factor for sepsis-associated organ failure and death (53) and higher PAI-1 levels have been reported in sepsis non-survivors on days 1, 4, and 8 (54). Previous studies have associated lower PAI-1 levels with weight loss (55). While lower PAI-1 levels in our study may be related to the lower body weight of sepsis survivors, they may also suggest that the processes of fibrinolysis are impaired in mouse sepsis survivors. Collectively, these findings support the previously proposed concept of *persistent inflammation-immunosuppression and catabolic syndrome* in sepsis survivors (14).

We also found that increased peripheral inflammation and innate immune hyporesponsiveness coexist with brain alterations, including dysregulation in cholinergic signaling and increased neuroinflammation in mouse sepsis survivors. Basal forebrain cholinergic neurons have been implicated in the regulation of cognitive functions, including attention, learning, and memory, and motivation, emotional states, and cerebral blood flow (23). Neurodegeneration of these neurons is directly implicated in cognitive impairment in Alzheimer's disease (23). Our results indicate a previously unrecognized vulnerability of basal forebrain cholinergic neurons among sepsis survivors, indicated by decreased numbers of ChAT-positive neuronal bodies in this area. Basal forebrain cholinergic neurons innervate the cortex (neocortex), hippocampus, and other forebrain

regions. In sepsis survivors, we observed a trend toward higher AChE activity and significantly increased *Ache* gene expression in the cortex. In addition, AChE activity was significantly increased in the cortex and *Ache* gene expression was unaltered in the hippocampus. Considering the role of AChE as the main enzyme degrading acetylcholine, these observations may reflect depletion of acetylcholine in the synaptic cleft and cholinergic hypofunction. We also found region-specific alterations in the gene expression of *Chrm1* (coding for M1 mAChR), a decrease in the hippocampus and no difference in cortex. Given the important role of forebrain cholinergic signaling in cognition, our observations suggest that brain cholinergic dysfunction can be an important underlying event contributing to the cognitive deficits in sepsis survivors. Accordingly, enhancing brain cholinergic signaling might be useful for treating cognitive deterioration in sepsis survivors.

Neuroinflammation is a major component in brain functional dysregulation in sepsis-associated encephalopathy (9). Microglia activation has been directly associated with a systemic inflammatory reaction in a case–control study which focused on the distribution of immunophenotype of microglia in brain tissue collected from patients with sepsis (11). Neuronal degeneration in the cortex and hippocampus, in parallel with increased inflammation, and blood brain barrier disruption has been reported as early as 12–24 h after CLP surgery in mice (56). In this study, we observed morphological alterations in microglia found in the cortex, indicative of activation, and associated with upregulated *Iba1* gene expression. In line with the role of activated microglia as a major source of proinflammatory cytokines, we found increased *Il1b* and *Il6* gene expression in the cortex and hippocampus. This neuroinflammation is accompanied by dysregulated cholinergic signaling in these brain areas, indicative of cholinergic hypofunction. Previously, brain cholinergic signaling has been shown to control neuroinflammation; increased cholinergic signaling suppresses microglial activation, and the release of proinflammatory cytokines (57). Therefore, it is plausible that cholinergic hypofunction and uncontrolled microglial activation in the cortex and hippocampus contributes to increased neuroinflammation among sepsis survivors. These two findings raise the intriguing question of whether decreased brain cholinergic tone contributes to the focal brain inflammation. It is known that exacerbated peripheral and brain inflammatory activation and cytokine release have deleterious effects on neurons (16). In a case–control study systemic infection and inflammation was associated with microglia activation in brain tissue from patients who died of sepsis (11). We and others have demonstrated brain microglial activation in the presence of increased levels of peripheral serum cytokines (16, 33, 58). Our *in vitro* data shed additional light on the relationship between activated microglia and impaired cholinergic signaling in the cortex; microglial activation has a profound impact on cholinergic components, including ChAT and AChE, the main enzymes associated with acetylcholine synthesis and degradation, respectively. Accordingly, this loss of cholinergic anti-inflammatory control on brain inflammation may be at least partially caused by products released by activated microglia contributing (*via* currently unknown mechanism) to cholinergic neurodegeneration.

Brain cholinergic M1 mAChR signaling also regulates peripheral inflammation (17, 26, 29, 31). Cortex areas and the hippocampus innervated by cholinergic pathways interact, directly or through multisynaptic pathways, with the dorsal vagal complex, the brainstem integrative center of the inflammatory reflex, a vagus nerve-based powerful regulator of peripheral immune responses and inflammation (17, 52, 59, 60). Our findings highlight an interplay between systemic and brain inflammation, which can result in neuronal damage of brain cholinergic neurons and impaired cholinergic neurotransmission in murine sepsis survivors. These findings together with the previously indicated role of brain cholinergic signaling in cognition and the control of peripheral and local inflammation suggests that cholinergic dysfunction may further facilitate inflammation and this reciprocal relationship may be an important underlying event contributing to cognitive impairment in sepsis survivors. Accordingly, brain cholinergic dysfunction may be a relevant therapeutic target for alleviating the chronic illness observed among sepsis survivors.

ETHICS STATEMENT

All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) of the Feinstein Institute for Medical Research, Northwell Health, Manhasset, NY, USA.

AUTHOR CONTRIBUTIONS

VAP, NZ, MA, KJT, and BD designed research; NZ, MEA, HHS, HLP, SIV-F, KRA, KRL, MN, MEA, and VP performed research; NZ, MEA, HP, KA, KL, PO, MN, CNM, PW, MA, SSC, KJT, and VAP analyzed and interpreted data; NZ and VAP wrote the article; SIV-F, KRL, CNM, PW, MA, BD, and KJT provided additional comments and contributed to finalizing the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01673/full#supplementary-material.

FIGURE S1 | Choline acetyltransferase and DAPI immunostaining in basal forebrain (scale bar = 100 μm).

FIGURE S2 | Cholinergic gene expression in cortex and hippocampus in sham-operated control mice **(A)** *Ache* gene expression. $P = 0.022$, Student's *t*-test, $n = 8$ per group. **(B)** *Chrm1* gene expression. $P = 0.0012$, Student's t-test, n = 8 per group.

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Exercise Prevents Enhanced Postoperative Neuroinflammation and Cognitive Decline and Rectifies the Gut Microbiome in a Rat Model of Metabolic Syndrome

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Introduction: Postoperative cognitive decline (PCD) can affect in excess of 10% of surgical patients and can be considerably higher with risk factors including advanced age, perioperative infection, and metabolic conditions such as obesity and insulin resistance. To define underlying pathophysiologic processes, we used animal models including a rat model of metabolic syndrome generated by breeding for a trait of low aerobic exercise tolerance. After 35 generations, the low capacity runner (LCR) rats differ 10-fold in their aerobic exercise capacity from high capacity runner (HCR) rats. The LCR rats respond to surgical procedure with an abnormal phenotype consisting of exaggerated and persistent PCD and failure to resolve neuroinflammation. We determined whether preoperative exercise can rectify the abnormal surgical phenotype.

Materials and methods: Following institutional approval of the protocol each of male LCR and male HCR rats were randomly assigned to four groups and subjected to isoflurane anesthesia and tibia fracture with internal fixation (surgery) or anesthesia alone (sham surgery) and to a preoperative exercise regimen that involved walking for 10 km on a treadmill over 6 weeks (exercise) or being placed on a stationary treadmill (no exercise). Feces were collected before and after exercise for assessment of gut microbiome. Three days following surgery or sham surgery the rats were tested for ability to recall a contextual aversive stimulus in a trace fear conditioning paradigm. Thereafter some rats were euthanized and the hippocampus harvested for analysis of inflammatory mediators. At 3 months, the remainder of the rats were tested for memory recall by the probe test in a Morris Water Maze.

Results: Postoperatively, LCR rats exhibited exaggerated cognitive decline both at 3 days and at 3 months that was prevented by preoperative exercise. Similarly, LCR rats had excessive postoperative neuroinflammation that was normalized by preoperative exercise. Diversity of the gut microbiome in the LCR rats improved after exercise.

Discussion: Preoperative exercise eliminated the metabolic syndrome risk for the abnormal surgical phenotype and was associated with a more diverse gut microbiome. Prehabilitation with exercise should be considered as a possible intervention to prevent exaggerated and persistent PCD in high-risk settings.

Keywords: preoperative exercise, postoperative cognitive decline, postoperative neuroinflammation, microbiome, metabolic syndrome

INTRODUCTION

Postoperative cognitive decline (PCD) is a devastating complication with long-lasting consequences especially in elderly surgical patients. PCD encompasses both the DSM V-defined Postoperative Delirium as well as the imprecise entity of postoperative cognitive dysfuction whose existence has been challenged (1). Devising strategies to limit the occurrence and/or severity of PCD will require a thorough understanding of the underlying pathophysiological mechanisms.

By interpreting data acquired from preclinical studies of PCD, we propose that aseptic peripheral trauma engages the innate immune response through cellular disruption and release of cytosol-located high molecular group box 1 protein (HMGB1) at the sites of tissue damage (2). HMGB1 initiates synthesis and release of proinflammatory cytokines through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in circulating bone marrow-derived monocytes (BM-DMs) (2). HMGB1 also upregulates monocyte chemoattractant protein-1 (MCP-1) in the central nervous system (2). Peripheral inflammation disrupts the blood–brain barrier, allowing CCR2-expressing BM-DMs to enter the hippocampus, attracted by its cognate ligand, MCP-1 (3). Together with the CNS-residing immunocompetent microglia that become activated (4), the BM-DMs generate a neuroinflammatory response that interferes with long-term potentiation, a form of synaptic plasticity required for learning and memory (5). In most settings, both inflammation and cognitive decline are short-lived as neural (6) and humoral (7) mechanisms promptly resolve the inflammation.

Patients with metabolic syndrome are particularly prone to develop PCD (8, 9). To further explore the reasons for this enhanced risk, we have used a validated animal model in which low capacity runner (LCR) rats, bred for their limited aerobic exercise capacity, exhibit each of visceral obesity, hypertension, hyperlipidemia, and insulin resistance, the cardinal features of the metabolic syndrome (10). After aseptic trauma, the LCR rats develop an exaggerated and more persistent form of cognitive decline (11) that is associated with a failure of resolution of inflammation (11).

Aerobic exercise training attenuates inflammation by altering signaling in innate immune cells (12–16). Exercise has also been shown to limit inflammation in type 2 diabetic patients (17). Therefore, we sought to determine whether exercise rectifies the neuroinflammatory response to surgical trauma in LCR rats and whether the exaggerated and persistent PCD can be mitigated. As an altered gut microbiome may be a driver for persistent inflammation (18) we also determined the effect of exercise on the diversity of the microbiome in LCR rats.

MATERIALS AND METHODS

Animals

All experimental procedures involving animals were approved by Institutional Animal Care and Use Committee of University of California, San Francisco (protocol no.: AN167062), and conformed to National Institute of Health guidelines (**Figure 1**). All animals were housed (two rats per cage) in saw dust-lined cages in an air-conditioned environment with 12-h light/dark cycles and were fed standard rodent chow and water *ad libitum*. LCR rats and high capacity runner (HCR) rats were developed by Koch and Britton using the 35th generation (19). Rats were randomly allocated to surgery or sham group before any procedure was undertaken and researchers were blinded to the group assignment during assessments, prior to the analysis phase.

Surgery

Under general anesthesia with 2.1% isoflurane in 0.30 FiO_2, rats underwent an open tibial fracture of the left hind paw with intramedullary fixation under aseptic surgical conditions as previously described (11, 20). Briefly, under sterile conditions a 20 G pin was inserted in the intramedullary canal of the left tibia, and osteotomy was performed after the periosteum was stripped. During the surgical procedure that lasted approximately 10 min, temperature was monitored and maintained at 37°C with the aid of warming pads (Harvard Apparatus, Holliston, MA, USA). Buprenorphine (0.1 mg/kg) was given subcutaneously to provide analgesia after the induction of anesthesia and before skin

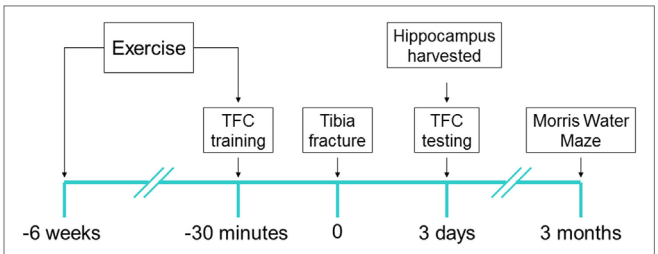

FIGURE 1 | Experimental design: each of low capacity runner and high capacity runner rats were randomly assigned into groups to receive interventions of exercise/no exercise and surgery/sham surgery. Exercise-assigned rats exercised on a treadmill for 6 weeks prior to surgery/sham surgery. 30 min prior to tibia fracture (surgery) under general anesthesia or sham surgery, the training session for trace fear conditioning (TFC) was performed and context testing was performed 72 h later (6–8/group). Immediately after TFC context testing rats were sacrificed and the brain was harvested. Separate cohorts (5–7/group) were tested for cognition 3 months after surgery using Morris Water Maze probe test.

incision. The sham rats were exposed to anesthesia and analgesia as above.

Exercise

Rats, both LCR as well as HCR, were exposed to a 6-week treadmill exercise protocol before surgery similar to Britton and Koch's exercise protocol with some modifications (10). During the first week of the protocol, rats were acclimated to treadmill exercise for gradually increasing duration each day beginning with 1 min at a velocity of 10 m/min and by day 5 rats exercised for 5 min. From week 2, rats exercised 5 days a week over 6 weeks with an identical exercise protocol for the same week gradually achieving 31.5 min at a speed of 20 m/min. Exercise animals ceased their training 48 h prior to further experimental procedures. The control (non-exercise) rats were placed on a non-moving treadmill daily for an equivalent duration to the time that the exercise rats spent on the moving treadmill.

Behavioral Studies

Trace Fear Conditioning

Trace fear conditioning was used to assess memory in rodents as previously described (11, 20). In brief, the training chamber is connected to a shock delivery system (Med Associates, St. Albans, VT, USA). During the training, rats were allowed to explore this context for 3 min after which they were presented with a conditional stimulus, an auditory cue (75–80 dB, 5 kHz,) for 20 s. Twenty seconds after termination of the auditory tone, the unconditional stimulus, a 2-s foot shock (0.8 mAmp) was administered. Rats were removed from the training chamber after an additional 30 s. Surgery was performed within 30 min after training. Three days later, rats were placed back into the same chamber ("context") in which it was trained but with neither tone nor shock. The memory of the learned fear was assessed by freezing behavior each 5 s during the 5 min observation time. The percentage of the observation period that the animal adopted freezing behavior was calculated using the formula $100 \times f/n$, where f is the number of freezing events per rat and n is the total number of observations per rat.

Morris Water Maze

Three months after surgery, rats were investigated in the Morris Water Maze as previously reported and now briefly described (11).

Cued Trials

The cued trial includes three training sessions with a visible platform by placing a visible yellow flag on top of the platform; the platform was relocated between each session.

Visuospatial Reference Memory

The platform is hidden in this trial. The rat was released from the assigned location while facing the wall of the tank. Six fixed locations were randomly selected to generate one long, one medium, and one short swim every session. Equal numbers of rats were randomly allocated to one of the four quadrants of platform locations in order to minimize any bias related to platform location. 90 s were set as a cutoff value. The rats were trained until they could locate the hidden platform in less than 15 s, on average, within a session.

Probe Trials

Immediately after the last training session (in which rats could locate the platform $\leq$ 15 s), the 60-s probe trial, reflected by memory retention for the hidden platform location, was performed with the platform removed from the tank. Time spent in the target quadrant, where the platform formerly resided, was recorded as the dwelling time. Swimming speed, and time spent in the target quadrant were analyzed using an EthoVision video tracking system (Noldus Instruments, Wageningen, Holland).

Inflammation

Hippocampal IL-6 Protein Expression

Three days after surgery, rats were sacrificed under isoflurane anesthesia. The hippocampus was harvested and stored at −80°C for further analysis. The hippocampus from one side was homogenized in cell lysis buffer (Cell Signaling Technology), mixed with phenylmethanesulfonyl fluoride (Cell Signaling Technology) and protease inhibitor (Thermo Fisher Scientific). Protein concentration was assayed with Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Interleukin IL-6 was measured using a commercially available ELISA kit following the manufacturer's instructions (R&D Systems).

Hippocampal Inflammatory Mediators mRNA Expression by Quantitative Polymerase Chain Reaction (q-PCR)

The hippocampus from the other side was harvested at 3 days after surgery was placed in RNAlater™ solution (Qiagen). RNA extraction, RNA-to-cDNA reverse transcription, and q-PCR were performed as previously described (20). Relative gene expression for IL-6, HMGB-1, MCP-1, Integrin alpha-X (Itgax), Netrin-1 (Ntn1), Arginase 1(Arg1), and Mannose receptor C type 2 (Mrc2), were calculated using the comparative threshold cycle ΔΔCt and housekeeping gene β-actin for normalization of gene expression. The mRNA levels for target genes are expressed as fold increases relative to LCR + sham + exercise group.

Gut Microbiome

Feces were collected from rats prior to exercise and after exercise. Samples were provided to Microbiome Core at University of California, San Francisco for DNA extraction, PCR amplification of the V4 hypervariable region of the 16sRNA gene, and DNA sequencing on the Illumina MiSeq. DNA was extracted from the samples using the MoBio PowerSoil DNA Isolation Kit according to the manufacturer's recommendations. For each sample, the PCR was amplified in triplicate using primer pairs that (i) targeted the V4 hypervariable region of the16srRNA gene, (ii) contained a unique barcode sequence to enable demultiplexing of pooled samples, and (iii) contained an adapter sequence that enables the amplicon to bind to the MiSeq flowcell. Amplicons were pooled in equimolar concentrations and sequenced on the Illumina MiSeq. Paired sequencing reads were quality filtered and demultiplexed using the QIIME software package before being assembled and processed further. Briefly, assembled sequencing

read pairs were binned into operational taxonomic units (OTUs) using a 97% similarity to the green genes database; reads that neither clustered to the green genes database nor were chimeric were removed from subsequent analyses. Sample read numbers were rarefied to the read number of the lowest sample after processing (88,048) resulting in a rarefied OTU table. From this OTU table downstream analysis was performed, and alpha and beta diversity indices were calculated.

Statistical Analysis

All data in this study were analyzed using Prism 6.0 (GraphPad Software, San Diego, CA, USA) and were expressed as mean ± SD. Statistical comparison was performed by a one-way ANOVA followed by Newman–Keuls multiple comparison test for *post hoc* analysis. Significance was set at $P < 0.05$.

RESULTS

Preoperative Exercise and Body Weight

As diet was not restricted exercise alone did not significantly alter body weight in the groups (n = 12–14/group) (**Figure 2**). The relative difference in body weight between the LCR and HCR rats that was present before exercise was sustained after exercise.

Preoperative Exercise Attenuated Exaggerated Acute Cognitive Decline in Metabolic Syndrome Rats

Acute postoperative memory, as assessed by percent of time spent freezing when returning to the same training context, was impaired in both HCR (80.1 ± 8.6 vs. 52 ± 4.3%, $P < 0.001$) and LCR (78.9 ± 10.9 vs. 40.5 ± 5.1%, $P < 0.001$) rats at 3 days after surgery (**Figure 3**). The decline in freezing time was greater in the LCR rats than the HCR rats (40.5 ± 5.1 vs. 52 ± 4.3%, $P < 0.05$), which is consistent with our previous report (11). Preoperative exercise rectified the exaggerated acute cognitive impairment in LCR rats (HCR + surgery + exercise vs. LCR + surgery + exercise, 51.3 ± 1.6 vs. 49.5 ± 3.1%, $P > 0.05$). Preoperative exercise in the LCR surgical group significantly improved the freezing time compared to the non-exercise LCR surgical group (49.5 ± 3.1 vs. 40.5 ± 5.1%, $P < 0.05$). Cohorts varied between 6 and 8/group.

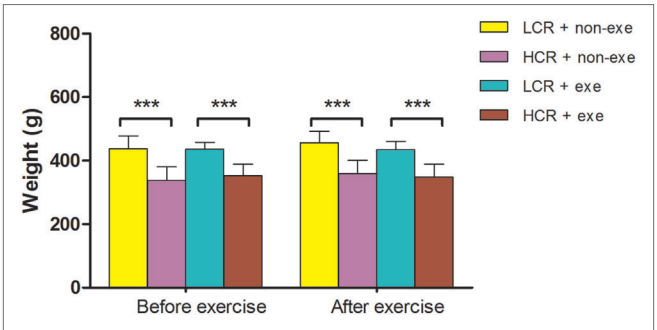

FIGURE 2 | Exercise and body weight: LCR and HCR rats were randomly assigned into four groups (n = 12–14) and trained with and without exercise. Exercise was performed 5 days per week for 6 weeks. Body weight (g) was recorded before exercise and immediately after exercise and is expressed as means ± SD, and analyzed by one-way ANOVA and Newman–Keuls *post hoc* analysis. ***$P < 0.001$ for comparisons shown.

Preoperative Exercise Attenuated Persistent Cognitive Decline in Metabolic Syndrome Rats

In the Morris Water Maze test, swimming speed was similar in all groups at 3 months after surgery (**Figure 4**). Long-term postoperative memory was assessed by dwelling time in the target quadrant in the probe trial of the Morris Water Maze test. The time that postoperative LCR rats spent in the quadrant in which the platform formerly resided was significantly shorter than for HCR rats (42.2 ± 1.9 vs. 60.2 ± 7.5%, $P < 0.01$), which is consistent with our previous report (11). Again, preoperative exercise rectified the persistent cognitive decline in LCR rats (HCR + surgery + exercise vs. LCR + surgery + exercise, 53.4 ± 7.5 vs. 63.3 ± 10.3%, $P > 0.05$). Preoperative exercise in the LCR surgical group significantly improved the dwell time compared to the non-exercise LCR surgical group (53.4 ± 7.5 vs. 42.2 ± 1.9% $P < 0.05$). Cohorts varied between 5 and 7/group.

Preoperative Exercise Prevented Abnormal Neuroinflammation in Metabolic Syndrome Rats

Three days after surgery, hippocampal proinflammatory cytokine, IL-6 protein expression (**Figure 5A**), was remarkably elevated in LCR and HCR surgical rats with greater elevation in LCR surgical rats than HCR surgical rats (45.72 ± 10.88 vs. 32.41 ± 1.79 pg/mg, $P < 0.001$) (**Figure 5**). The mRNA levels for IL-6 (**Figure 5B**), HMGB1 (**Figure 5C**), MCP-1 (**Figure 5D**), Itgax (**Figure 5E**), and Netrin-1 (**Figure 5F**) were each increased in the surgical rats with LCR surgical rats exhibiting a significantly greater increase than

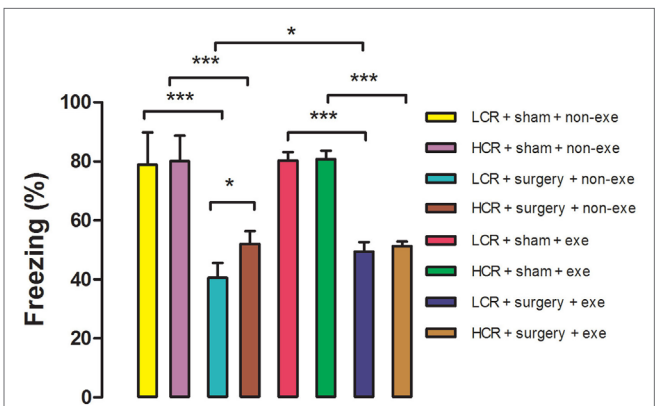

FIGURE 3 | Preoperative exercise attenuated exaggerated acute cognitive decline in metabolic syndrome: 3 days after exercise (or no exercise) training, HCR and LCR rats (6–8/group) were subjected to preoperative training in a trace fear conditioning (TFC) paradigm. Surgery and sham surgery were performed with 30 min after training. Freezing behavior (represented as the as the % of time spent freezing over a 5 min observation period) was tested in the same TFC training context at 3 days after surgery. Percent time freezing is expressed as means ± SD, and analyzed by one-way ANOVA and Newman–Keuls *post hoc* analysis. *$P < 0.05$ and ***$P < 0.001$ for comparisons shown.

the HCR surgical rats (6.2- vs. 3.1-fold increase for IL-6, $P < 0.05$; 2.5- vs. 1.5-fold increase for HMGB-1, $P < 0.001$; 6.6- vs. 3.7-fold increase for MCP-1, $P < 0.01$; 7.7- vs. 4-fold increase for Itgax, $P < 0.001$; 2.8- vs. 2.1-fold increase for Netrin-1, $P < 0.01$). The mRNA levels of the pro-resolving mediators Arg1 (**Figure 5G**) and Mrc2 (**Figure 5H**) were significantly increased in both HCR and LCR rats although the increase in HCR rats was greater than in the LCR rats (2.5- vs. 1.7-fold increase for Arg1, $P < 0.05$; 3.8- vs. 1.8-fold increase for Mrc2, $P < 0.05$). For each of the genes, preoperative exercise eliminated the differences in the surgery-induced changes in the proinflammatory (**Figures 5A–F**) mediators. Exercise significantly attenuated the increase in pro-inflammatory cytokines in the postoperative LCR rats (IL-6 protein expression 24.97 ± 4.76 vs. 45.72 ± 10.88 pg/mg, $P < 0.001$; IL-6 mRNA 2.3- vs. 6.2-fold increase, $P < 0.01$; HMGB-1 mRNA 1.2- vs. 2.5-fold, $P < 0.001$; MCP-1 mRNA 2.9- vs. 6.6-fold, $P < 0.001$; Itgax mRNA 2.2- vs. 7.7-fold, $P < 0.001$; Netrin-1 mRNA 1.5- vs. 2.8-fold, $P < 0.001$). Cohorts varied between 5 and 7/group.

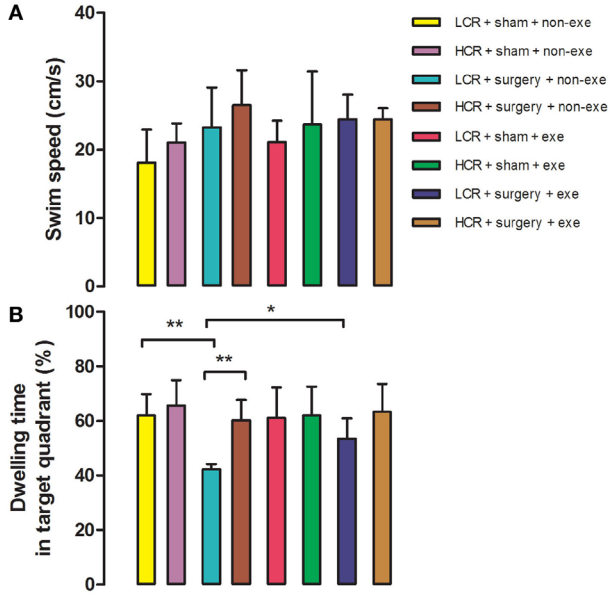

FIGURE 4 | *Preoperative* exercise attenuated persistent cognitive decline in metabolic syndrome rats: 3 months after surgery, HCR and LCR rats were trained and tested in the Morris Water Maze paradigm. Swimming speed **(A)** and target quadrant dwelling time **(B)** as a percent of the total observation period of 60 s were recorded and expressed as mean ± SD, and analyzed by one-way ANOVA and Newman–Keuls *post hoc* analysis. $^{**}P < 0.01$ for comparisons shown.

Preoperative Exercise Rectified Abnormal Microbiome LCR Rats

The α diversity of the gut microbiome in the LCR rats ($n = 6$/group) was significantly improved after exercise (**Figure 6**) while no change was noted after exercise in the HCR rats (data not shown) (**Figure 6**). Exercise significantly improved the β diversity in both the LCR and HCR groups and significantly altered the abundance of two of the major phyla, Firmicutes and Bacteroidetes in the HCR rats (See Supplementary Material).

DISCUSSION

Major Findings

In LCR rats, preoperative exercise attenuated both the exaggerated acute postoperative decrease in freezing time at day

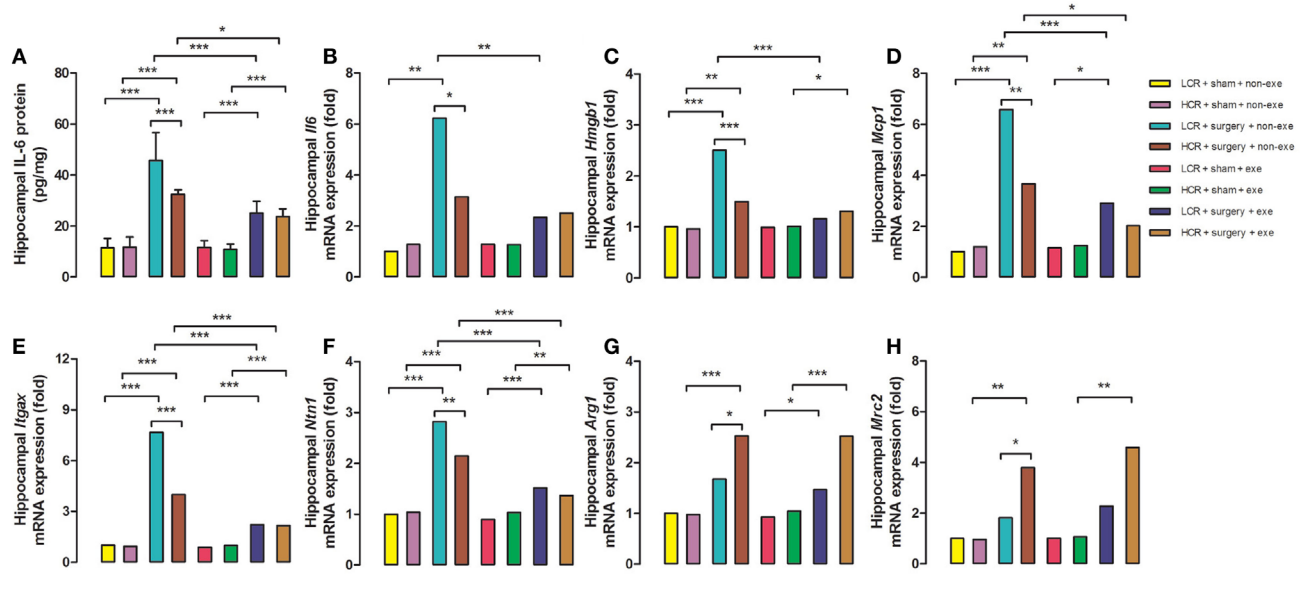

FIGURE 5 | Preoperative exercise prevented abnormal neuroinflammation in metabolic syndrome rats: 3 days after surgery, the hippocampus was harvested from each group ($n = 5$–7/group). Protein expression of IL-6 **(A)** and mRNA expression of IL-6 **(B)**, HMGB-1 **(C)**, MCP-1 **(D)**, Itgax **(E)**, Netrin-1 **(F)**, Arg1 **(G)**, and Mrc2 **(H)** were measured. The data were expressed as means ± SD, and analyzed by one-way ANOVA and Newman–Keuls *post hoc* analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for comparisons shown.

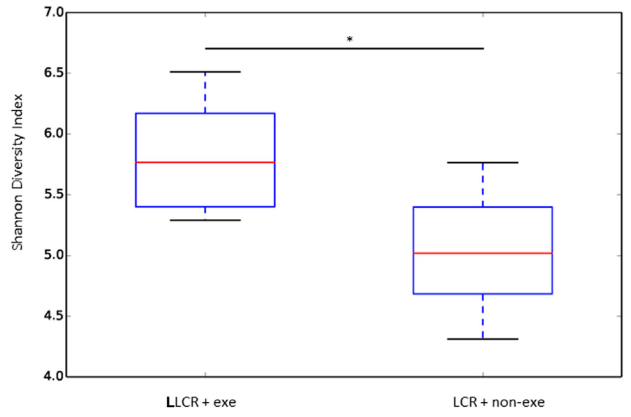

FIGURE 6 | Exercise rectified α diversity of the fecal microbiome in metabolic syndrome rats: low capacity runner rats were divided into exercise and non-exercise groups ($n = 6$) and stools were collected after 6 weeks of exercise or no exercise for microbiome assessment. The α diversity of the fecal microbiome was calculated by the Shannon method. Data are expressed as box and whisker plots with median flanked by first and third quartiles and variance bars showing the extremes. Groups were compared by *t*-test. $^*P < 0.03$.

3 (**Figure 3**) as well as the decrement in recall (dwelling time) in the Morris Water Maze probe trial performed three months postoperatively (**Figure 4**). The enhanced neuroinflammatory response to surgical trauma was attenuated by exercise in the LCR rats (**Figures 5A–F**). The α diversity of the gut microbiome was significantly improved by exercise in the LCR rats (**Figure 6**). In aggregate these data suggest that preoperative exercise can rectify the neuroinflammatory response to surgery resulting in an elimination of the exaggerated and persistent PCD. The exercise-induced improvement in postoperative neuroinflammation and cognition in the LCR rats was associated with a significant improvement in gut dysbiosis.

Metabolic Syndrome, Inflammation, Effects of Exercise

Over the past two decades Britton and Koch produced two cohorts of rats that, at generation 35, differ more than 10-fold in their treadmill running capacity at peak exhaustion (21). The rapid fatigability contributes to the sedentary behavior of the LCR rats (22) that is the likely antecedent (23) to their development of obesity, hypertension, hyperlipidemia, and insulin resistance (24). Earlier, we had demonstrated that the LCR rats exhibit a more exaggerated and persistent cognitive decline (11) that is associated with failure of both the neural and humoral inflammation-resolution pathways (20). The hyperinflammatory states that are observed in obesity and insulin resistance have been termed "metainflammation" which is thought to contribute to the comorbidities that occur in these metabolically deranged states (25). The failure to appropriately resolve inflammation in the LCR rats results in a relative increased neuroinflammatory response (**Figures 5A–F**) and a relative decrease in the pro-resolving response (**Figures 5G,H**) when compared to the HCR rats. These altered postoperative neuroinflammatory responses were corrected by preoperative exercise in the LCR rats (**Figures 5A–H**).

Gut Microbiome, Obesity, Inflammation, and Exercise

Characteristics of a healthy microbiome include community stability and increased species diversity. Obesity is associated with reduced bacterial diversity in gut microbiome (26). Similarly, a lower level of bacterial diversity was also observed in the setting of the metabolic syndrome (27) and the altered microbiome may contribute to hyperlipidemia (28). As the gut microbiome can perform functions that modulate the immune system (29) it is unsurprising that changes in the microbiome can influence the inflammatory response. The gut microbiome is in a state of dynamic homeostasis with the immune and nervous systems (30, 31). In a mouse model of Alzheimer's disease, change in peripheral cytokines and microglia occurred pari passu with alteration of the gut microbiome induced by broad-spectrum oral antibiotics (32). With advanced age there is less diversity in the gut microbiome (33) and the chronic low-grade inflammatory response associated with aging, referred to as "inflammaging," has recently been linked to age-related microbiome changes (34).

Several recent mice studies have now shown a direct link between exercise and beneficial changes in the gut microbiome. The dysbiosis present in high fat diet-induced obese mice was corrected by exercise (35). In another high-fat diet mouse study, exercise did induce microbiota changes but these alterations were not the same as those produced by the high-fat diet (36).

In the current study there was a significant improvement in the α (**Figure 6**) and the β diversity (see Figure S1 in Supplementary Material) of the gut microbiome following exercise in the LCR rats. An α diversity index (such as the Shannon index) reflects the species diversity in a community and takes into account not only species richness but also the relative abundances of different species (evenness). Thus, the α diversity index indicates how many types and how equally the microbiome is present in one subject. The β diversity represents a difference in species diversity between different environments and illustrates exchange or similarity of species between compared environments.

Our data, which demonstrate that an exercise-induced improvement in the diversity of the gut microbiome of the LCR rats (**Figure 6**) is associated with a normalization of the neuroinflammatory response (**Figure 5**), are corroborated by previous reports that a less diverse gut microbiome is associated with a hyperinflammatory state (34). Interestingly, the exercise-induced changes in the taxonomic profiles that were observed in both the LCR and HCR rats involved an increase Firmicutes and a decrease in Bacteroidetes although the change was greater and statistically significant for the HCR rats (see Figure S2 in Supplementary Material). Re-assuringly, the direction of these exercise-induced changes in the taxonomic profiles of these two major phyla was similar to that observed in post-exercise high-fat diet obese mice (36). The sequence data are provided in Table S1 in Supplementary Material.

Caveats

Although we had observed exercise-induced changes in the gut microbiome that were associated with less postoperative neuroinflammation and cognitive decline, it is not possible to ascribe a cause and effect relationship between the improvement in the diversity of the gut microbiome and the attenuation of the surgical phenotype. In order to address the causal nature of the abnormal microbiome in the LCR rats for the exaggerated and persistent surgical phenotype, future studies will need to address the direct effects of altering the microbiome, possibly by diet or fecal transplantation.

Implications

PCD imposes significant costs on patients, their families and caretakers, and more broadly on society as a whole. A recent meta-analysis revealed that in older patients, delirium is associated with an increased risk of death, institutionalization, and dementia (37). Understanding the postoperative pathophysiological mechanisms and developing an intervention with the potential to reduce PCD have the potential for significant clinical impact. Of course, not all patients awaiting surgery will be able to exercise, either because of their physical condition or because the surgery is urgent. However, even these patients may benefit from the knowledge of the changes that exercise induces in the innate immune system as these could be a target for a different type of intervention designed, for example, to enhance the diversity and stability of the gut microbiome.

ETHICS STATEMENT

All experimental procedures involving animals were approved by Institutional Animal Care and Use Committee (IACUC) of University of California, San Francisco (protocol no.: AN167062), and conformed to National Institute of Health guidelines.

AUTHOR CONTRIBUTIONS

XF and YU contributed equally as first authors. XF performed the behavioral studies that were performed at 3 days and 3 months and contributed to the writing of the manuscript. YU collected samples, analyzed samples, and participated in the microbiome analysis and contributed to the writing of the manuscript. LK generated the LCR and HCR rats. SB generated the LCR and HCR rats. JH performed exercise studies. DL performed the exercise studies. MM conceived of the experiment, obtained the funding, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01768/full#supplementary-material.

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ORIGINAL RESEARCH
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Anesthesia and Surgery Impair Blood–Brain Barrier and Cognitive Function in Mice

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Blood–brain barrier (BBB) dysfunction, e.g., increase in BBB permeability, has been reported to contribute to cognitive impairment. However, the effects of anesthesia and surgery on BBB permeability, the underlying mechanisms, and associated cognitive function remain largely to be determined. Here, we assessed the effects of surgery (laparotomy) under 1.4% isoflurane anesthesia (anesthesia/surgery) for 2 h on BBB permeability, levels of junction proteins and cognitive function in both 9- and 18-month-old wild-type mice and 9-month-old interleukin (IL)-6 knockout mice. BBB permeability was determined by dextran tracer (immunohistochemistry imaging and spectrophotometric quantification), and protein levels were measured by Western blot and cognitive function was assessed by using both Morris water maze and Barnes maze. We found that the anesthesia/surgery increased mouse BBB permeability to 10-kDa dextran, but not to 70-kDa dextran, in an IL-6-dependent and age-associated manner. In addition, the anesthesia/surgery induced an age-associated increase in blood IL-6 level. Cognitive impairment was detected in 18-month-old, but not 9-month-old, mice after the anesthesia/surgery. Finally, the anesthesia/surgery decreased the levels of β-catenin and tight junction protein claudin, occludin and ZO-1, but not adherent junction protein VE-cadherin, E-cadherin, and p120-catenin. These data demonstrate that we have established a system to study the effects of perioperative factors, including anesthesia and surgery, on BBB and cognitive function. The results suggest that the anesthesia/surgery might induce an age-associated BBB dysfunction and cognitive impairment in mice. These findings would promote mechanistic studies of postoperative cognitive impairment, including postoperative delirium.

Keywords: anesthesia/surgery, interleukin-6, blood–brain barrier, age, cognition

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INTRODUCTION

Anesthesia and surgery have been shown to induce postoperative cognitive impairment in rodents [(1–10), reviewed in Ref. (11)]. However, the underlying mechanism by which the anesthesia/surgery causes cognitive impairment remains largely to be determined.

The blood–brain barrier (BBB) is composed of blood vessels with endothelial cells with extremely low rates of paracellular vesicular transport (12) and transcellular vesicular transport (transcytosis) [(13–15), reviewed in Ref. (16)]. The BBB provides a safe environment for the central nervous system (CNS), which is free of pathogens and toxins in normal conditions [reviewed in Ref. (17)].

Compromised BBB function might contribute to the cognitive impairment, brain damage, and neurodegenerative disorders, e.g., Alzheimer's disease [(18, 19), reviewed in Ref. (16, 20, 21)]. Specifically, peripheral inflammation can profoundly affect the function of the CNS, including cognitive impairment and delirium, through a compromised BBB (22–24). Stranahan et al. reported that BBB dysfunction (e.g., increase in BBB permeability) might induce cognitive impairment in obese and diabetic patients by allowing macrophage infiltration and trafficking of interleukin (IL)-1β from peripheral monocytes to the brain (25). Zhang et al. suggested that BBB dysfunction caused CNS inflammation and cognitive impairment (26). Based on these findings, we aimed to assess the effects of the anesthesia/surgery on BBB permeability, the potential underlying mechanisms and cognitive function in the current studies.

Our previous studies have shown that surgery under local anesthesia induces an age-dependent cognitive impairment in mice (6, 7), and anesthetic isoflurane (27) and sevoflurane (28, 29) can increase IL-6 level. Therefore, we also sought to determine whether the anesthesia/surgery-induced change in BBB permeability and cognitive function was associated with the advanced age and dependent on IL-6. Finally, we compared the effects of anesthesia/surgery on the levels of β-catenin, tight junction proteins (claudin, occludin, and ZO-1) [(30), reviewed in Ref. (16)], and adherent junction protein (VE-cadherin, E-cadherin, and p120-catenin) [(31), reviewed in Ref. (16)].

The objective of the current studies was to determine the effects of the anesthesia/surgery on BBB permeability, cognitive function and blood IL-6 levels, and brain levels of tight junction and adherent junction proteins in adult and older mice, which could lead to the further mechanistic investigation of postoperative cognitive impairment, including postoperative delirium. Our hypothesis is that the anesthesia/surgery induces an age-associated and IL-6 dependent increase in BBB permeability, leading to cognitive impairment in mice.

MATERIALS AND METHODS

Mice Surgery and Treatment

All experiments were performed in accordance with the National Institutes of Health guidelines and regulations. The animal protocol was approved by the Massachusetts General Hospital (Boston, MA, USA) Standing Committee on the Use of Animals in Research and Teaching. Efforts were made to minimize the number of animals used. Wild-type C57BL/6J female mice (9-month-old, The Jackson Laboratory, Bar Harbor, ME, USA; and 18-month-old, National Institute of Aging, Bethesda, MD, USA), and 9-month-old IL-6 gene knockout female mice (B6.129S2-Il6tm1Kopf/J, The Jackson Laboratory) were used in the studies.

We only used female mice in the current studies because our previous studies showed that the female mice were more vulnerable to the development of cognitive impairment following the same anesthesia/surgery (32). Mice were randomly assigned to the anesthesia/surgery group or control group by weight. The 18-month-old mice are only available through National Institute of Aging, thus cannot be purchased from same company that provides the 9-month-old mice. Mice in the anesthesia/surgery group had a simple laparotomy under isoflurane anesthesia using the methods described in our previous studies (10, 33). Specifically, we anesthetized each of the mice using 1.4% isoflurane in 100% oxygen in a transparent acrylic chamber. Fifteen minutes after the induction, we moved the mouse out of the chamber. Isoflurane anesthesia was maintained *via* a cone device and one 16-G needle was inserted into the cone near the nose of the mouse to monitor the concentration of isoflurane. We made a longitudinal midline incision from the xiphoid to the 0.5 cm proximal pubic symphysis on the skin, abdominal muscles, and peritoneum. We then sutured the incision layer by layer with 5–0 Vicryl thread. We applied EMLA cream (2.5% lidocaine and 2.5% prilocaine) to the incision site at the end of the procedure, and then every 8 h until the euthanasia of the mice, to treat the pain associated with the incision. The procedure for each mouse usually lasted about 10 min and we put the mouse back into the anesthesia chamber for up to 2 h to receive the rest of the anesthesia consisting of 1.4% isoflurane in 100% oxygen. We used this method because surgery could potentiate the anesthesia neurotoxicity and such combination of anesthesia and surgery had been shown to induce cognitive impairment (10, 33). We maintained the rectal temperature of the mice at 37 ± 0.5°C during the anesthesia/surgery by using DC Temperature Control System (FHC, Bowdoinham, ME, USA). We returned the mice back to their home cage with food and water available *ad libitum* after recovering from the anesthesia. The mice in the control group were placed in their home cages with regular room air for 2 h, which was consistent with the condition of non-surgery patients. Our previous studies found that neither the surgery (6, 7) nor anesthesia with 1.4% isoflurane (34) significantly disturbed the blood pressure and blood gas values of the mice. EMLA could treat the pain associated with the surgery in the mice (6, 7). The treatment of IL-6 antibody was performed as described in previous studies with modification (4). Specifically, each of the 18-month-old mice received the 10 μg IL-6 antibody (eBioscence Inc., San Diego, CA, USA, Cat. Number: 16-7061) at 18 h before the anesthesia/surgery *via* tail vein injection under brief anesthesia (1.4% isoflurane for 5 min). The control mice received saline. We used a single injection of IL-6 antibody because a single injection of TNF-α antibody had been shown to mitigate the surgery-induced cognitive impairment in mice in the studies by Terrando et al. (35).

Brain Tissue Harvest

We harvested the brain tissues of the mice for the dextran imaging studies and spectrophotometer quantification of dextran. We harvested both cortex and hippocampus for the Western blot analysis. Each of the mice was perfused with phosphate-buffered saline (PBS) for the spectrophotometric quantification of dextran.

Specifically, mouse received thoracotomy under brief anesthesia (1.4% isoflurane for 5 min), we inserted a needle to left ventricular of the heart and perfused slowly with sufficient amount of PBS (five times with 30 ml PBS each time) until the PBS exiting from right heart became colorless. We then decapitated the head of each of the mice and harvested the brain tissues. We stored the brain tissues in a −80°C freezer for future analysis.

Dextran Imaging Studies to Detect BBB Permeability

Dextran was used to measure BBB permeability as described in previous studies with modifications (15). Specifically, 6 h after the anesthesia/surgery, each of the mice was briefly anesthetized with 1.4% isoflurane for 5 min for the injection of dextran. 100 µl 10-kDa dextran tetramethylrhodamine lysine fixable (4 mg/ml, Catalog number: D3312, Invitrogen) was injected into the mouse through the tail vein. Ten minutes after the injection, each of the mice was decapitated; brain tissues (e.g., cortex) were harvested and fixed by 4% paraformaldehyde overnight at 4°C. The brain tissues (e.g., cortex) were cryopreserved in 30% sucrose and frozen in TissueTek OCT (Sakura). The immunohistochemistry to detect BBB permeability was performed as described in the previous studies (15) with modifications. Frozen sections (the thickness of each section: 12 µm) from mouse brain hemispheres were cut and used for the immunohistochemistry staining. These sections were postfixed in 4% PFA at room temperature (20–25°C) for 15 min, washed in PBS, and were blocked with 2% albumin from bovine serum, permeabilized with 0.5% Triton X-100, and incubated with isolectin B4 (1:200; Catalog number: I21411, Molecular Probes, San Francisco, CA, USA) for the immunohistochemistry imaging of blood vessels. Dextran itself has fluorescence for the detection of immunohistochemistry imaging. Finally, the sections were analyzed in mounting medium (Catalog number: ab104139; Abcam, Cambridge, MA, USA) under a 40× objective lens of the fluorescence microscope and photos of the sections were taken. These images were analyzed manually with ImageJ (National Institutes of Health, Bethesda, MD, USA). Coronal cortical sections (12 µm) of the same rostrocaudal position were used for the analysis. The same threshold was used and the same acquisition parameters were applied to all images. An investigator who was blind to the experimental design manually measured the level (combined with area and intensity) of the dextran tracer-positive area found outside the vessel using ImageJ. For each mouse, we obtained five slices from front, middle, and posterior section of the brain tissue (e.g., cortex). Two images per slice were taken. Therefore, 30 images (3 section × 5 slices × 2 images) of each mouse were counted. There were three mice in control group and three mice in the anesthesia/surgery group. Thus, the quantification of the images was based on these 90 images per group.

Spectrophotometric Quantification of Dextran to Detect BBB Permeability

Spectrophotometric quantification of 10-kDa fluoro-ruby-dextran tracer (555/580) (4 mg/ml, Catalog number: D1817, Invitrogen) from cortex extracts was used as described in previous studies (15, 36). The 100 µl dextran tracer was injected into each of the mice *via* the tail vein under brief anesthesia (1.4% isoflurane for 5 min) at 1 h after the anesthesia/surgery. 16 h after the injection of the tracer, each of the mice was cardiac perfused as described in the Section "Brain Tissues Harvest." Brain tissues (e.g., cortex) were then harvested and the 10-kDa fluoro-ruby-dextran tracer (555/580) from cortex extracts was determined by Spectrophotometric measurement.

Enzyme-Linked Immunosorbent Assay (ELISA) Determination of IL-6

The mouse IL-6 Immunoassay kit (Catalog number: M6000B, R&D Systems) was used to determine the levels of IL-6 in mouse blood as described by the protocol associated with the immunoassay kit. Briefly, a monoclonal antibody specific for mouse IL-6 was coated onto the microplates. Wells were incubated for 2 h at room temperature with test samples (serum) and washed for five times. Then, 100 µl of mouse IL-6 conjugate was added to each well and incubated for another 2 h and we repeated the washing. Finally, wells were incubated in 100 µl of substrate solution for 30 min and stopped with stop solution (100 µl). Determination of the optical density of each well was set at 450 nm and corrected at 570 nm.

Western Blot Analysis

Western blot analysis was performed using the methods described in our previous studies (8). Cortex and hippocampus tissues were harvested from 18-month-old mice at 6, 12, and 24 h after the anesthesia/surgery. Anti-β-catenin antibody (92 kDa, Cat: #9562, 1:1,000 dilution, Cell signaling, Danvers, MA, USA), anti-phosphorylated β-catenin antibody (Ser33/37/Thr41, 92 kDa, Cat: #9561, 1:1,000 dilution, Cell signaling), anti-claudin-1 antibody (19 kDa, Cat: ab15098, 1:1,000 dilution, Abcam, Cambridge, MA, USA), anti-occludin antibody (59 kDa, Cat: ab167161, 1:1,000 dilution, Abcam), anti-ZO-1 antibody (250 kDa, Cat: PA5-28858, 1:500 dilution, Thermo Fisher Scientific, Rockford, IL, USA), and anti-VE-cadherin (115 kDa, Cat: ab33168, 1 µg/ml, Abcam), anti-E-cadherin antibody (135 kDa, Cat: #5296, 1:1,000 dilution, Cell signaling) were used to detect the level of the proteins in the cortex and hippocampus of the 18-month-old mice. Samples in different groups were loaded and β-actin was used to normalize (e.g., determining the ratio of β-catenin to β-actin amount) protein levels and control for loading differences in the total protein amount. The quantification of the Western blot was performed as described in previous study (8), we presented changes in protein levels in hippocampus or cortex treated with anesthesia/surgery as a percentage of those in the control group. 100% of protein level changes refer to control levels for the purpose of comparison to experimental conditions. Signal intensity was analyzed using a Bio-Rad (Hercules, CA, USA) image program.

Morris Water Maze (MWM)

Morris water maze was carried out to assess spatial learning and memory function as previously described (7, 28). The blind procedure was not possible in the MWM studies because of the appearance of the abdominal wound in the mice. Briefly, all mice were trained to swim to a hidden platform in four trials per day for 6 days (days 1–6) starting 3 days after the anesthesia/

surgery (**Figure 1**). Each of the mice was given 90 s to find the platform and allowed to stay there for 15 s before being removed from the platform. Mice would be guided to the platform and allowed to stay on the platform for 15 s if they could not find the platform within 90 s. The platform was placed in the target quadrant in trials within one MWM test, but the starting points were random for each mouse. We measured the time it took for each mouse to reach the platform (escape latency) and used this as the learning score. On the seventh day, we repeated the training same as the previous training (days 1–6). Two hours after the training, we removed the platform and assessed the memory of each mouse by measuring the number of times the mouse crossed the platform area with the same cue (memory score). We compared the learning and memory score and swimming speed between the mice in the control group and the anesthesia/surgery group.

Barnes Maze

Barnes maze test was performed by using the methods described in other studies (37–40) with modifications. The blind procedure was not possible in the Barnes maze studies because of the appearance of the abdominal wound in the mice. Barnes maze with a circular open platform (about 90-cm diameter) was located in a quiet area. It had 20 equally spaced holes (one of these holes connects with a small dark recessed chamber called escape box) and was surrounded by a dark curtain with four simple colored-paper shapes (square, circle, triangle, and star) as markers (Stoelting, Wood Dale, IL, USA). A video camera that could capture the entire platform was right above the platform and connected to the Any-Maze animal tracking system software (Stoelting Co., Wood Dale, IL, USA) as described in previous studies (38, 41, 42). The movement parameters, including escape latency (the total time to find the escape box), escape distance (the total path length of distance traveled), escape speed (mean speed), escape errors (the total error holes searched), and time in target quadrant [the percentage of time spent in the target quadrant, time in target quadrant (%)], of the mouse before finding the escape box in both training and test were monitored and analyzed *via* the video camera. The Barnes maze test in the current studies included Barnes maze training test (days 12–15 after the anesthesia/surgery) and Barnes maze test (16 days after the anesthesia/surgery) (**Figure 1**). On day 11 after the anesthesia/surgery, all of the mice were habituated to the maze. The mouse was placed in the escape box for 2 min and then placed directly in the hole that led to the escape box for another 4 min. Finally, the mouse was placed under a bucket in the center of the circular platform and motivated to escape under the bright light (200 W) and noise (85 dB) stimulation. Mouse was gently guided to the hole connecting to the escape box when it did not go into the escape box 3 min after the light and noise stimulation. Immediately after the mouse entered the tunnel between the hole and the escape box, the buzzer was turned off. Each mouse was allowed to remain in the escape box for 1 min and then removed and placed back to the home cage.

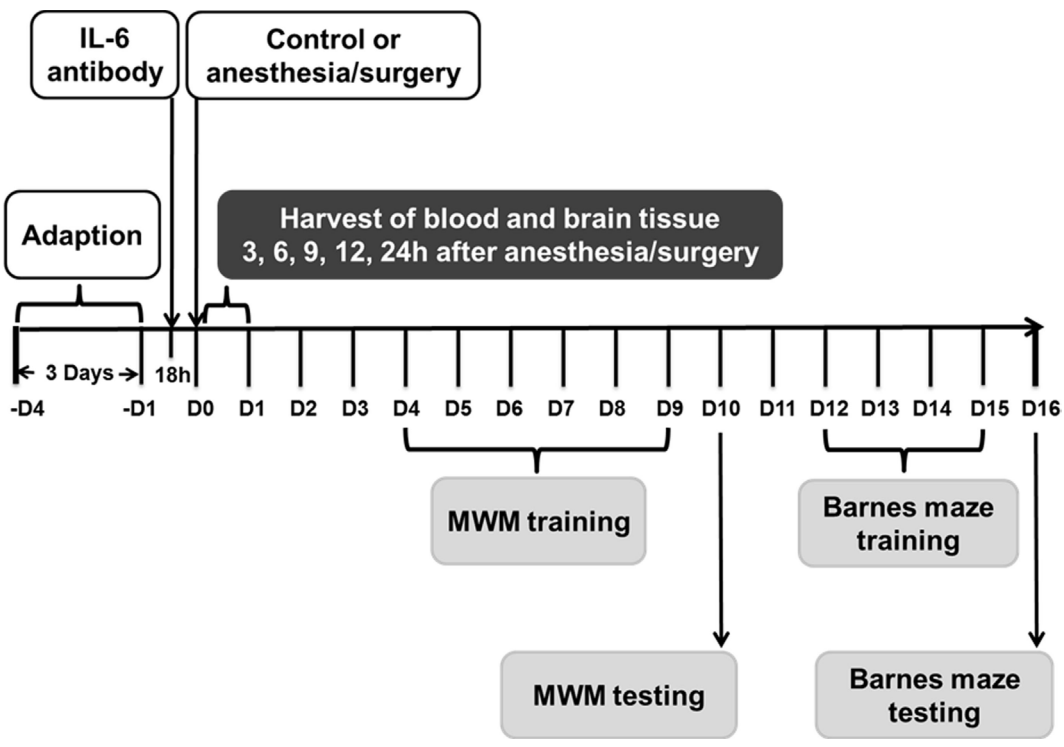

FIGURE 1 | Experimental design. Nine- and eighteen-month-old mice received the anesthesia/surgery or control condition. The blood and brain tissues were harvested at various time points after the anesthesia/surgery. Different group of mice were used for MWM and Barnes maze studies. Interleukin (IL)-6 antibody was used in the intervention studies. MWM, Morris water maze.

The Barnes maze training test (days 12–15 after the anesthesia/surgery) consisted of two trials (3 min each trial and 15 min between the trials) for 4 days. In each trial, the mouse was placed under a bucket in the center of the circular platform for 10 s and was allowed to escape under the same stimulation of light and aversive noise. Once reaching the escape box, the mouse was allowed to remain in the escape box for 1 min. The mouse was then removed and placed back to the home cage for 15 min of rest period before returning back for another trial. Between each test, the Barnes maze was cleaned with 75% alcohol solution to avoid olfactory cues. After the training period, the mice had the Barnes maze test on day 16 after the anesthesia/surgery (**Figure 1**). The increases in escape latency, escape distance, escape errors, and decrease in time in target quadrant in the Barnes maze suggests cognitive impairment of the mice (37–40). The decreases in escape speed suggest impairment of locomotor activity. We used the percentage of time in the target quadrant rather than the target preference in the current studies according to the previous studies (41, 43).

Statistics

Data of escape latency, escape distance, escape speed, escape errors, and time in target quadrant were expressed as means ± SEM. Data of other variables were expressed as means ± SD. The number of samples was 3 per group for ELISA studies, 4–5 per group for the spectrophotometer quantification of dextran, 90 per group for the immunohistochemistry imaging study, 10 per group for behavior test, and 6 per group for the Western blot studies. In the Barnes maze and Water maze studies, interaction between time and group factors in a two-way ANOVA with repeated measurements was used to analyze the difference of memory curves (e.g., based on escape latency). *Post hoc* analyses (Bonferroni) were used to compare the difference in all behavior test parameters for each testing day. In the biochemistry studies, one-way ANOVA or two-way ANOVA was used to determine the interaction between group and treatment on the levels of β-catenin, claudin, occludin, ZO-1, VE-cadherin, E-cadherin, and p120-catenin followed by Bonferroni test for the comparisons. All the protein levels were presented as a percentage of those of the control group. *P*-values less than 0.05 were considered statistically significant. Prism 6 software (Graph Pad Software, Inc., La Jolla, CA, USA) was used to analyze the data.

RESULTS

Anesthesia/Surgery Increases Extravascular Dextran Level in Mouse Brain in Age-Associated Manner

The details of the experimental design and performance were summarized in **Figure 1**. We employed immunohistochemistry staining of blood vessels (green color) and 10-kDa dextran (red color) to determine whether the abdominal surgery under isoflurane anesthesia (anesthesia/surgery) could increase the BBB permeability in mice. As compared to control condition (**Figure 2A**, first row), the anesthesia/surgery (**Figure 2A**, third row) increased the extravascular 10-kDa dextran level in the brain tissues of the 9-month-old mice. The quantification of the immunohistochemistry image showed that the anesthesia/surgery (**Figure 2B**, gray bar) increased the extravascular 10-kDa dextran level in the brain tissues of mice as compared to the control condition (**Figure 2B**, white bar) ($P < 0.001$, one-way ANOVA and Bonferroni test). These data suggest that the anesthesia/surgery was able to increase BBB permeability of 10-kDa dextran in the 9-month-old mice.

We then assessed the effects of the anesthesia/surgery on extravascular 10-kDa dextran level in the brain tissues of the 18-month-old mice. We found that the anesthesia/surgery induced a greater increase in the extravascular 10-kDa dextran level in the brain tissues of the 18-month-old mice (**Figure 2A**, fourth row) as compared to the control condition (**Figure 2A** second row) or the anesthesia/surgery in the 9-month-old mice (**Figure 2A**, third row). The quantification of the immunohistochemistry images showed that the anesthesia/surgery in 18-month-old mice (**Figure 2B**, net bar) increased the extravascular 10-kDa dextran level in the brain tissues of mice as compared to the control condition (**Figure 2B**, black bar) ($P < 0.001$, one-way ANOVA and Bonferroni test) or the anesthesia/surgery condition in the 9-month-old mice (**Figure 2B**, gray bar) ($P < 0.01$, one-way ANOVA and Bonferroni test). These data suggest that the anesthesia/surgery might induce a greater BBB permeability of 10-kDa dextran in the 18-month-old mice.

Finally, we found that the treatment with IL-6 antibody in the 18-month-old mice (**Figure 2A**, fifth row, and **Figure 2B**, dot bar) was able to attenuate the anesthesia/surgery-induced increase in BBB permeability of 10-kDa dextran (**Figure 2A**, fourth row, and **Figure 2B**, net bar). Anesthesia only in the 18-month-old mice did not significantly increase the BBB permeability of 10-kDa dextran (**Figure 2C**) as compared to the control condition in the 18-month-old mice (**Figure 2A**, second row). This image represents one mouse, but the study was repeated in another two mice. Moreover, we employed an IL-6 KO mouse to further assess the effects of IL-6 on the anesthesia/surgery-induced increase in BBB permeability. We found that there was no significant difference in extravascular 10-kDa dextran level in the brain tissues of the 9-month-old IL-6 knockout mouse between control condition and anesthesia/surgery condition (**Figure 2D**). The image represents one mouse, but the studies were repeated in another mouse. Taken together, these data suggest that the anesthesia/surgery-induced increase in BBB permeability to 10-kDa dextran would be dependent on IL-6 level.

Notably, the same anesthesia/surgery did not significantly increase BBB permeability to 70-kDa dextran in 9- or 18-month-old mice (**Figure 2E**). The image represents one mouse, but the study was repeated in another two mice. These data suggest that the anesthesia/surgery may only increase BBB permeability to 10-kDa, but not 70-kDa, dextran.

Finally, the findings that the anesthesia/surgery did not increase the BBB permeability to 10-kDa dextran in IL-6 KO mice and in the wild-type mice pre-treated with IL-6 antibody suggest that the perfusion itself would not increase the BBB permeability to 10-kDa dextran.

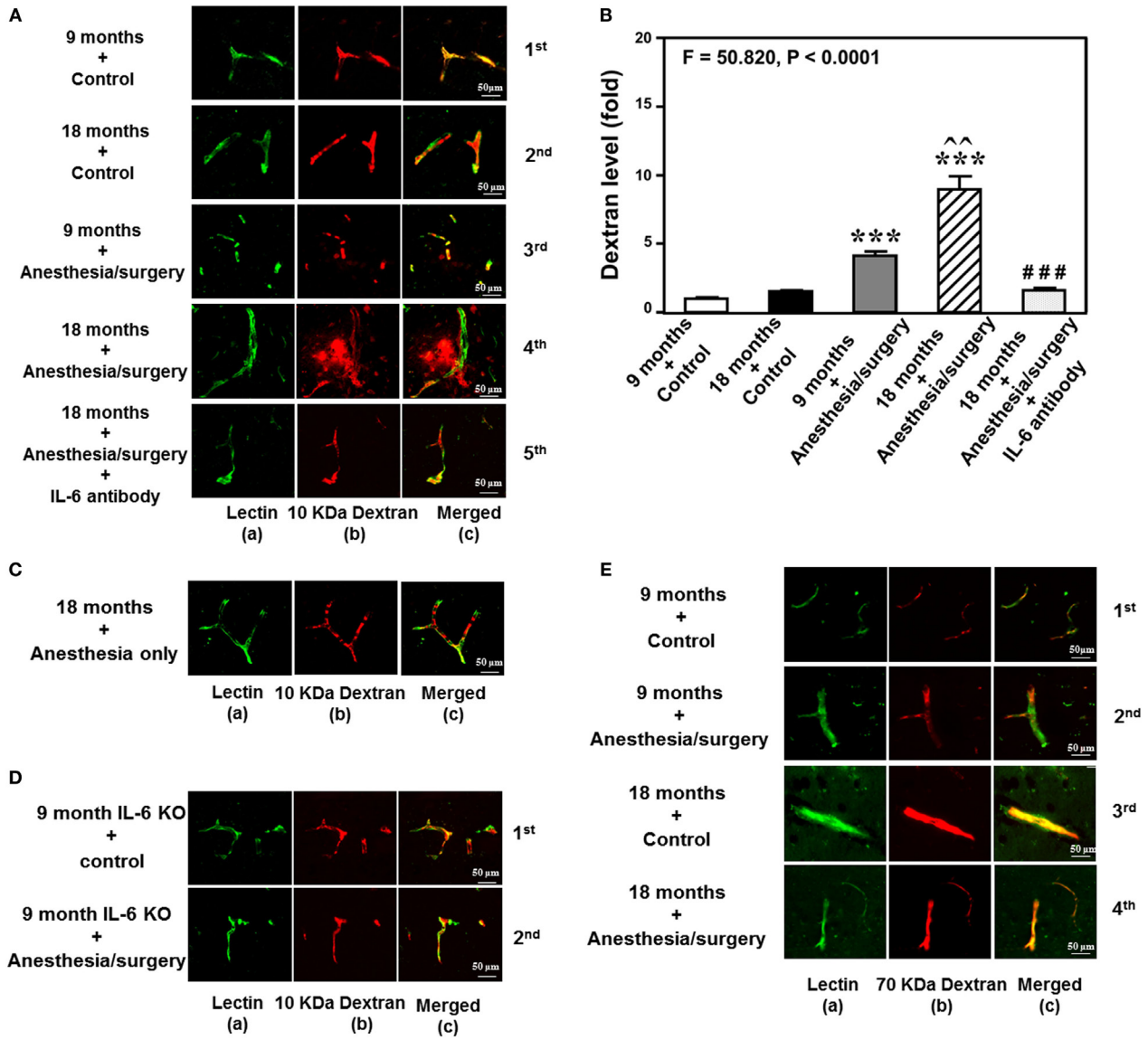

FIGURE 2 | Anesthesia/surgery increases blood–brain barrier permeability of dextran in mice in an age-associated manner. **(A)** Immunostaining of blood vessels (lectin, green, column *a*) and dextran (10-kDa dextran, red, column *b*) of the brain section following the control condition in 9-month-old mice (the first row), the control condition in 18-month-old mice (the second row), the anesthesia/surgery in 9-month-old mice (the third row), the anesthesia/surgery in 18-month-old mice (the fourth row), and the anesthesia/surgery in 18-month-old mice pre-treated with IL-6 antibody (the fifth row). Column *c* is the merged image of columns *a* and *b*. The red spots (non-overlap area) in column *c* indicate the dextran that is not inside the blood vessel (extravascular dextran). *N* = total of 90 slides from 3 mice in each group. **(B)** Quantification of the immunostaining images shows that the anesthesia/surgery in 9-month-old mice (gray bar) increases the extravascular dextran level in the mouse brain tissues as compared to that in the control condition in 9-month-old mice (white bar). The anesthesia/surgery in 18-month-old mice (net bar) increases extravascular dextran level in the mouse brain as compared to that in the control condition in 18-month-old mice (black bar) and that in anesthesia/surgery condition in 9-month-old-mice (gray bar). Treatment with IL-6 antibody (dot bar) attenuates the anesthesia/surgery-induced increase in the extravascular dextran level in the brain tissues of 18-month-old mice (net bar). **(C)** Immunostaining of blood vessels (lectin, green, column *a*) and dextran (10-kDa dextran, red, column *b*) of the brain section following anesthesia only in one 18-month-old mouse. Column *c* is the merged image of columns *a* and *b*. Anesthesia only does not increase the extravascular dextran level in the brain tissues of the 18-month-old mouse. The image represents one mouse. The studies were repeated twice in another two mice. **(D)** Immunostaining of blood vessels (lectin, green, column *a*) and dextran (10-kDa dextran, red, column *b*) of the brain section following anesthesia/surgery in one 9-month-old IL-6 gene knockout mouse. Column *c* is the merged image of columns *a* and *b*. The red spots (non-overlap area) in column *c* indicate the dextran that is not inside the blood vessel (extravascular dextran). Anesthesia/surgery does not significantly increase the extravascular dextran level in the brain tissues of the 9-month-old IL-6 gene knockout mouse. The study was repeated in another mouse. **(E)** Immunostaining of blood vessels (lectin, green color, column *a*) and 70-kDa dextran (red, column *b*) of the brain section following the control condition in 9-month-old mice (the first row), the anesthesia/surgery in 9-month-old mice (the second row), the control condition in 18-month-old mice (the third row), and the anesthesia/surgery in 18-month-old mice (the fourth row). Column *c* is the merged image of columns *a* and *b*. The red spots (non-overlap area) in column *c* indicate the 70-kDa dextran that is not inside the blood vessel (extravascular dextran). The anesthesia/surgery does not significantly increase the extravascular 70-kDa dextran level in the brain tissues of the 9-month-old mouse and 18-month-old mouse as compared to control condition. The image represents one mouse. The studies were repeated in another two mice. IL, interleukin.

Anesthesia/Surgery Increases Extravascular Level of Dextran in Mouse Brain

Next, we performed spectrophotometer quantification to further assess whether anesthesia/surgery could increase the extravascular level of 10-kDa dextran in brain tissues of mice. We found that the anesthesia/surgery increased the extravascular level of 10-kDa dextran in the brain tissues of 9-month-old mice (**Figure 3**, gray bar) as compared to the control condition in the 9-month-old mice (**Figure 3**, white bar) ($P < 0.01$, one-way ANOVA and Bonferroni test). Similarly, the anesthesia/surgery increased the extravascular level of 10-kDa dextran in the brain tissues of 18-month-old mice (**Figure 3**, net bar) as compared to the control condition in the 18-month-old mice (**Figure 3**, black bar) ($P < 0.01$, one-way ANOVA and Bonferroni test). However, anesthesia/surgery did not increase the extravascular level of 10-kDa dextran in the brain tissues of 9-month-old IL-6 KO mice (**Figure 3** dot bar versus gray bar versus white bar) ($P < 0.001$, one-way ANOVA and Bonferroni test). These data further suggest that the anesthesia/surgery is able to increase BBB permeability and the effects could be dependent on IL-6 level.

Anesthesia/Surgery Increases Blood IL-6 Level in Mice

Given the findings that the anesthesia/surgery might induce an IL-6-dependent increase in BBB permeability, next we asked whether the anesthesia/surgery could increase blood IL-6 level in the mice. We found that the anesthesia/surgery significantly increased blood IL-6 level as compared to control condition (*left two bars*) at 3 (*middle two bars*) and 6 (*right two bars*) hours after the anesthesia/surgery in both 9-month-old (*black* and *net bar*) and 18-month-old (*gray* and *dot bar*) mice ($F = 13.36$, $P = 0.0001$, one-way ANOVA, **Figure 4**). Note that the anesthesia/surgery-induced a larger increase in blood IL-6 levels in the 18-month-old mice as compared to that in the 9-month-old mice at 3 h after the anesthesia/surgery (*gray bar* versus *black bar*, $P < 0.05$). The anesthesia/surgery also induced a greater increase in blood IL-6 levels in the 18-month-old mice as compared to that in the 9-month-old mice at 6 h after the anesthesia/surgery (*net bar* versus *dot bar*). However, the difference did not reach to a statistically significant level. These data suggest that the anesthesia/surgery could induce an age-associated increase in blood IL-6 level in the mice, which would contribute to the anesthesia/surgery-induced increase in BBB permeability in the brain tissues of the mice.

Anesthesia/Surgery Induces Cognitive Impairment in an Age-Associated Manner

Given the findings that the anesthesia/surgery increased BBB permeability in an age-associated manner, next we investigated the functional relevance of these findings. Two-way ANOVA with repeated measurement showed no interaction of treatment (control condition and anesthesia/surgery) and time (days 1–7) on escape latency in the 9-month-old mice (**Figure 5A**, $F = 0.300$, $P = 0.937$). The anesthesia/surgery did not significantly change the platform crossing times (**Figure 5C**,

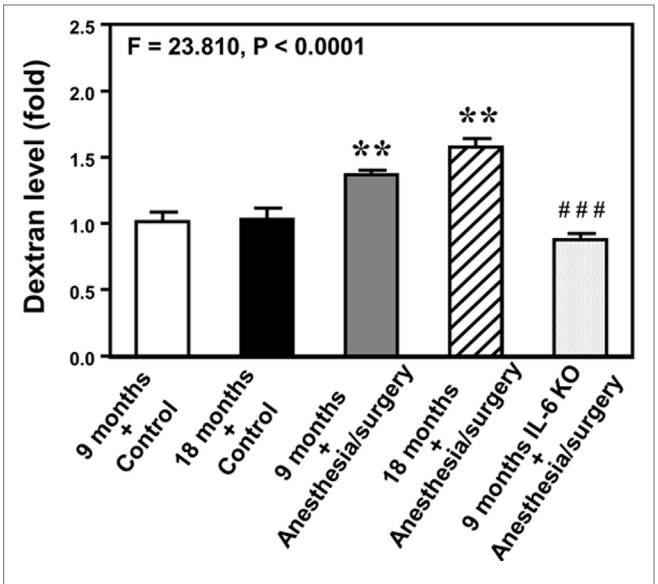

FIGURE 3 | Anesthesia/surgery increases blood–brain barrier permeability of dextran in mice. Spectrophotometric quantification of brain dextran (10 kDa) level. The anesthesia/surgery in 9-month-old mice (gray bar) increases brain dextran level as compared to that in the control condition of 9-month-old mice (white bar). Anesthesia/surgery in 18-month-old mice (net bar) increases brain dextran level as compared to that in the control condition of 18-month-old mice (black bar). Knockout of interleukin (IL)-6 gene (dot bar versus gray bar) attenuates the anesthesia/surgery-induced increase in dextran levels. $N = 4$ in the control condition group and $N = 5$ in the anesthesia/surgery group.

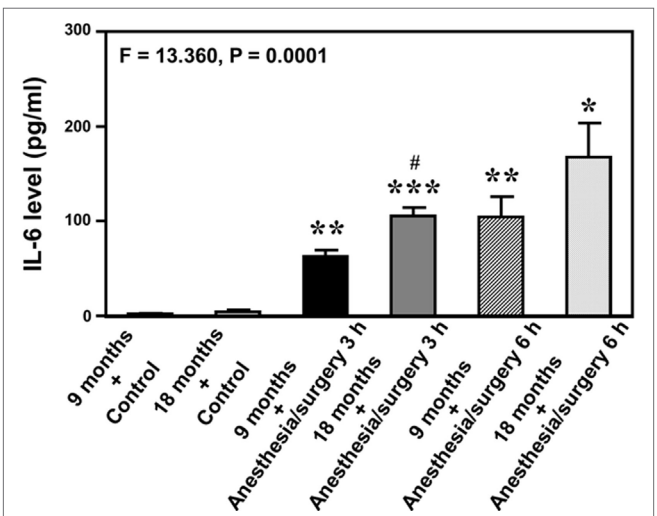

FIGURE 4 | Anesthesia/surgery increases blood IL-6 level in 9- and 18-month-old mice. The anesthesia/surgery increases blood IL-6 level in 9-month-old (black bar and net bar) and 18-month-old (gray bar and dot bar) mice at 3 (middle two bars) and 6 (right two bars) hours after the anesthesia/surgery as compared to the control condition (left two bars). The anesthesia and surgery induces a significant increase in blood IL-6 levels in the 18-month-old mice as compared to that in the 9-month-old mice at 3 and 6 h after the anesthesia/surgery. $N = 3$ in the control condition group and $N = 3$ in the anesthesia/surgery group. IL, interleukin.

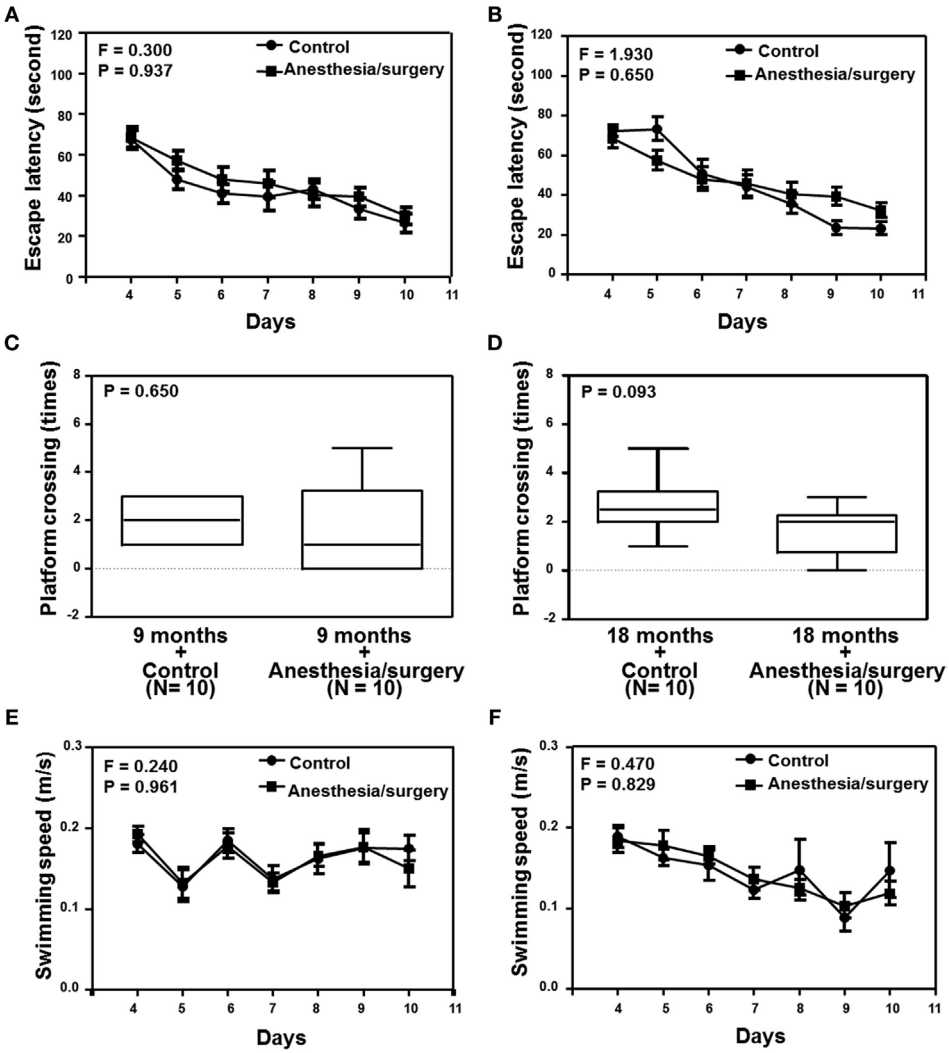

FIGURE 5 | Effects of anesthesia/surgery on cognitive function in 9- and 18-month-old mice tested in MWM. The anesthesia/surgery does not significantly change the escape latency **(A)**, platform crossing times **(C)**, and swimming speed **(E)** of MWM as compared to the control condition in the 9-month-old mice. The anesthesia/surgery does not significantly change the escape latency **(B)** and swimming speed **(F)** of MWM as compared to the control condition in the 18-month-old mice. However, the anesthesia/surgery induces a borderline increase ($P = 0.093$) in platform crossing times **(D)** of MWM as compared to the control condition in the 18-month-old mice. $N = 10$ in the control condition group and $N = 10$ in the anesthesia/surgery group. MWM, Morris water maze.

$P = 0.464$, Mann–Whitney test) or swimming speed (**Figure 5E**, $F = 0.240$, $P = 0.961$, two-way ANOVA with repeated measurement) as compared to the control condition in the 9-month-old mice. Two-way ANOVA with repeated measurement showed no interaction of treatment (control condition and anesthesia/surgery) and time (days 1–7) on escape latency in the 18-month-old mice (**Figure 5B**, $F = 1.930$, $P = 0.650$). The anesthesia/surgery induced a borderline increase in the platform crossing times (**Figure 5D**, $P = 0.093$, Mann–Whitney test) as compared to the control condition in the 18-month-old mice. The anesthesia/surgery did not significantly change the swimming speed (**Figure 5F**, $F = 0.240$, $P = 0.961$, two-way ANOVA with repeated measurement) as compared to the control condition in the 18-month-old mice.

We found that the anesthesia/surgery did not significantly change the escape latency (**Figure 6A**), escape distance (**Figure 6C**), escape speed (**Figure 6E**), escape errors (**Figure 6G**), and time in target quadrant (**Figure 6I**) as compared to the control condition in the 9-month-old mice. However, the anesthesia/surgery significantly increased the escape latency (**Figure 6B**, $P = 0.0296$, Student's t-test) and escape distance (**Figure 6D**, $P < 0.0001$, Student's t-test) as compared to the control condition in the 18-month-old mice. The anesthesia/surgery did not significantly change the escape speed (**Figure 6F**), escape errors (**Figure 6H**), and time in target quadrant (**Figure 6J**) as compared to the control condition in the 18-month-old mice. Taken together, these data suggest that the anesthesia/surgery could induce an age-associated cognitive impairment in mice.

Anesthesia/Surgery Decreases Levels of Cell Junction Proteins

Previous studies in rodents have shown that BBB permeability is associated with the alteration of cell junction protein in cerebral endothelial cells [reviewed in Ref. (16)]. Given the findings that the anesthesia/surgery was able to increase BBB permeability to small, but not big, molecules, next, we compared the effects of the anesthesia/surgery on the levels of β-catenin, tight junction

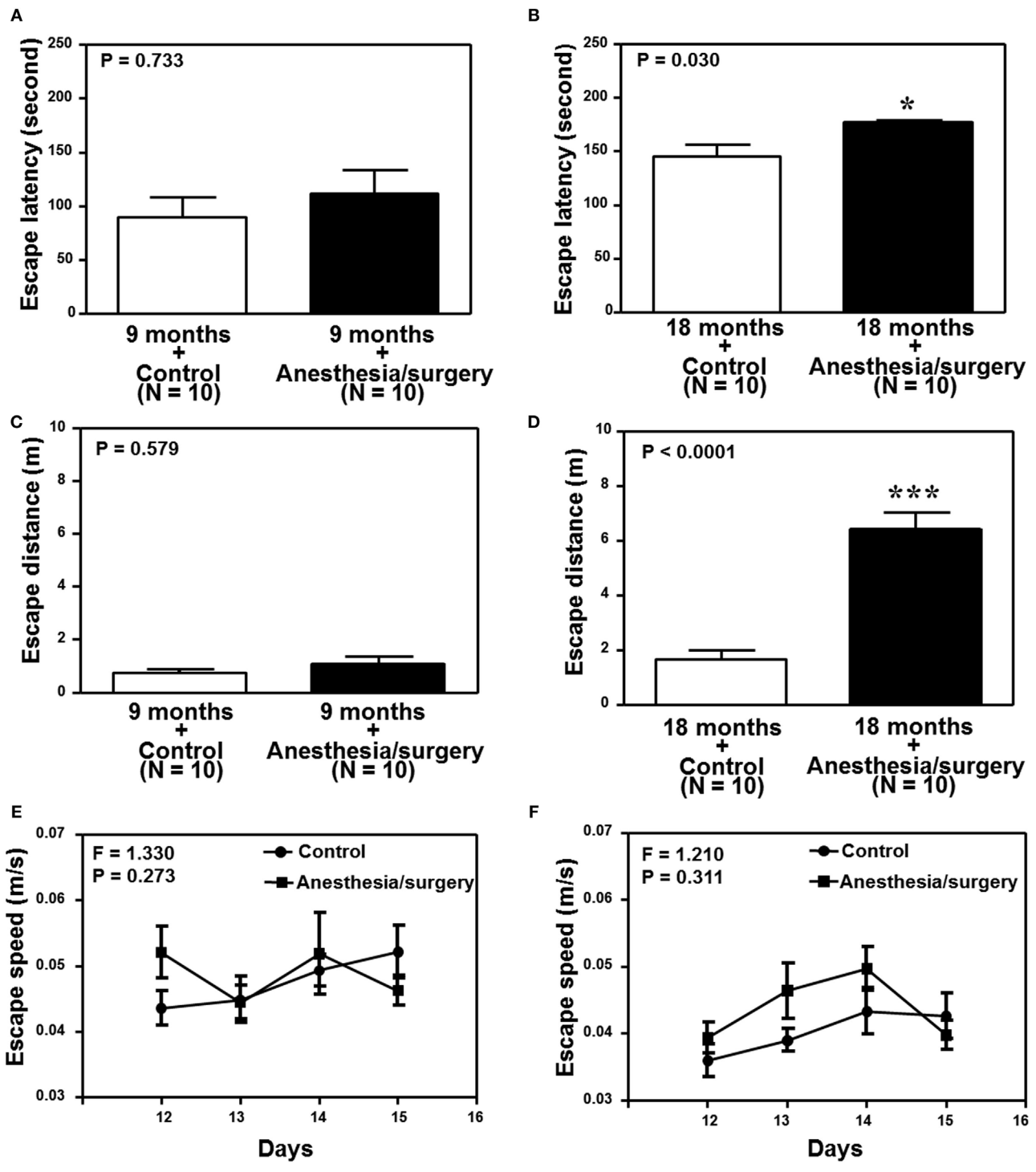

FIGURE 6 | Continued

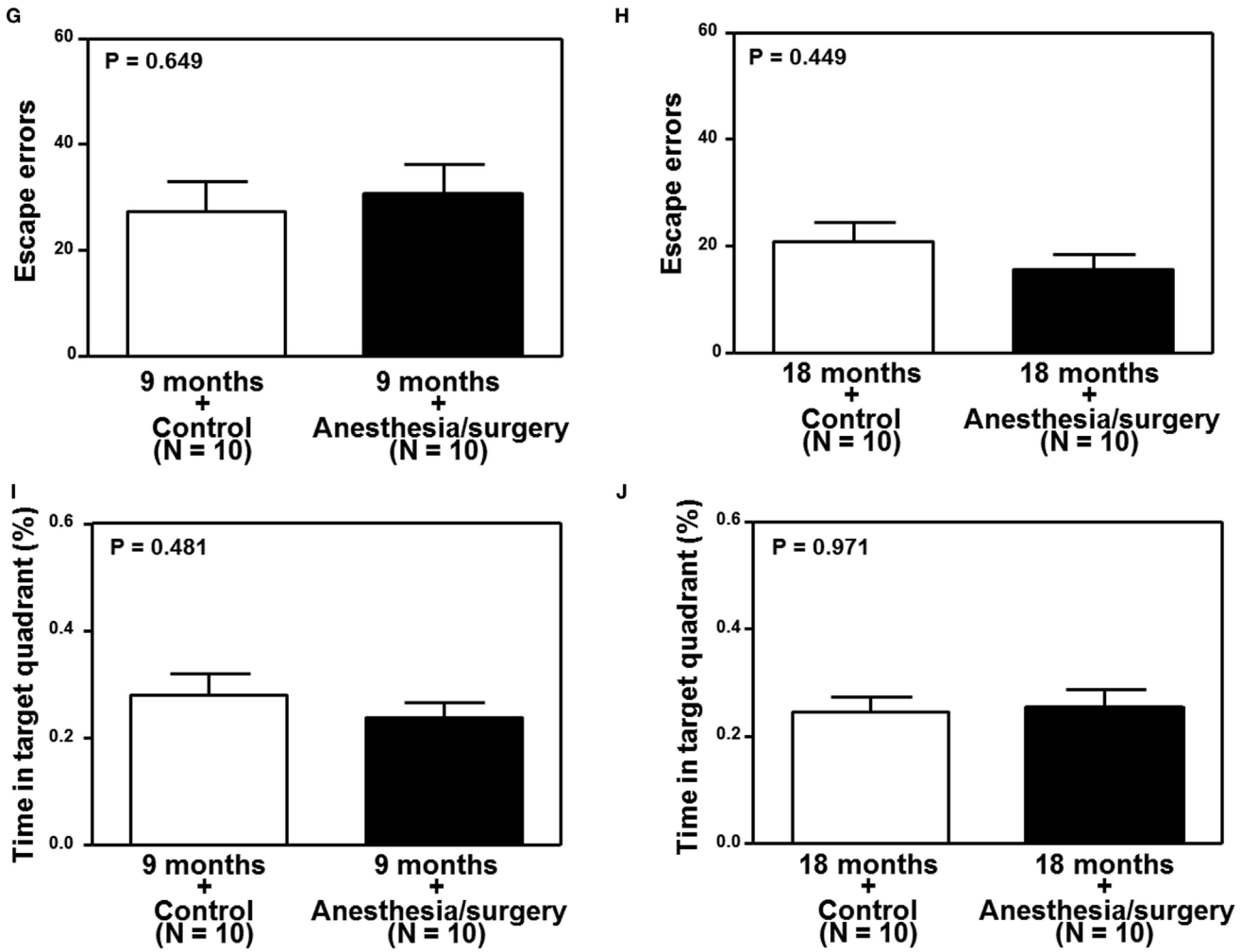

FIGURE 6 | Effects of anesthesia/surgery on cognitive function in 9- and 18-month-old mice tested in Barnes maze. The anesthesia/surgery does not significantly change the escape latency **(A)**, escape distance **(C)**, escape speed **(E)**, escape errors **(G)**, and time in target quadrant **(I)** of Barnes maze as compared to the control condition in the 9-month-old mice. However, in the 18-month-old mice, the anesthesia/surgery significantly increases escape latency **(B)** and escape distance **(D)** of Barnes maze as compared to the control condition. The anesthesia/surgery does not significantly change the escape speed **(F)**, escape errors **(H)**, and time in target quadrant **(J)** of Barnes maze as compared to the control condition in the 18-month-old mice. $N = 10$ in the control condition group and $N = 10$ in the anesthesia/surgery group.

proteins claudin, occludin, and ZO-1, and adherent junction proteins VE-cadherin, E-cadherin, and p120-catenin in cortex and hippocampus of 18-month-old mice. Quantitative Western blot showed that the anesthesia/surgery significantly decreased the level of β-catenin in cortex ($F = 15.280$, $P < 0.0001$, one-way ANOVA, **Figures 7A,B**) and hippocampus ($F = 39.090$, $P < 0.0001$, one-way ANOVA, **Figures 7C,D**) as compared to control condition at 6, 12, and 24 h after the anesthesia/surgery in 18-month-old mice. Moreover, quantitative Western blot showed that the anesthesia/surgery increased the phosphorylated β-catenin levels at 6, 12, and 24 h after the anesthesia/surgery as compared to control condition ($F = 5.233$, $P = 0.011$, one-way ANOVA, **Figures 7E,F**). Note that the antibody (92 kDa, Cat: #9561, 1:1,000 dilution, Cell signaling, Danvers, MA, USA) we used specifically detected the phosphorylated β-catenin (Ser33/37/Thr41) levels in the cytosol (44, 45). Finally, the anesthesia/surgery decreased levels of claudin as compared to control condition at 6, 12, and 24 h after the anesthesia/surgery in hippocampus of 18-month-old mice ($F = 40.170$, $P < 0.0001$, one-way ANOVA, **Figures 7G,H**).

Interestingly, the anesthesia/surgery did not significantly change the levels of the adherent junction proteins (VE-cadherin, E-cadherin, and p120-catenin) in cortex or hippocampus of 18-month-old mice as compared to the control condition at 6, 12, and 24 h after the anesthesia/surgery (Figure S1 in Supplementary Material). These data suggest that the anesthesia/surgery may specifically regulate the levels of tight junction proteins but not adherent junction proteins in present experiments.

IL-6 Antibody and KO of IL-6 Attenuates the Anesthesia/Surgery-Induced Reduction in the Levels of Cell Junction Proteins

The 18-month-old mice received IL-6 antibody 18 h before the anesthesia/surgery and the hippocampus tissues were harvested 24 h after the anesthesia/surgery. Quantitative Western blot showed that the treatment of IL-6 antibody significantly attenuated the anesthesia/surgery-induced reduction in the levels of β-catenin ($F = 7.420$, $P = 0.009$, two-way ANOVA, **Figures 8A,B**), claudin ($F = 10.310$, $P = 0.009$, two-way ANOVA, **Figures 8C,D**), occludin ($F = 10.340$, $P = 0.009$, two-way ANOVA, **Figures 8E,F**), and ZO-1 ($F = 23.280$, $P = 0.0007$, two-way ANOVA, **Figures 8G,H**)

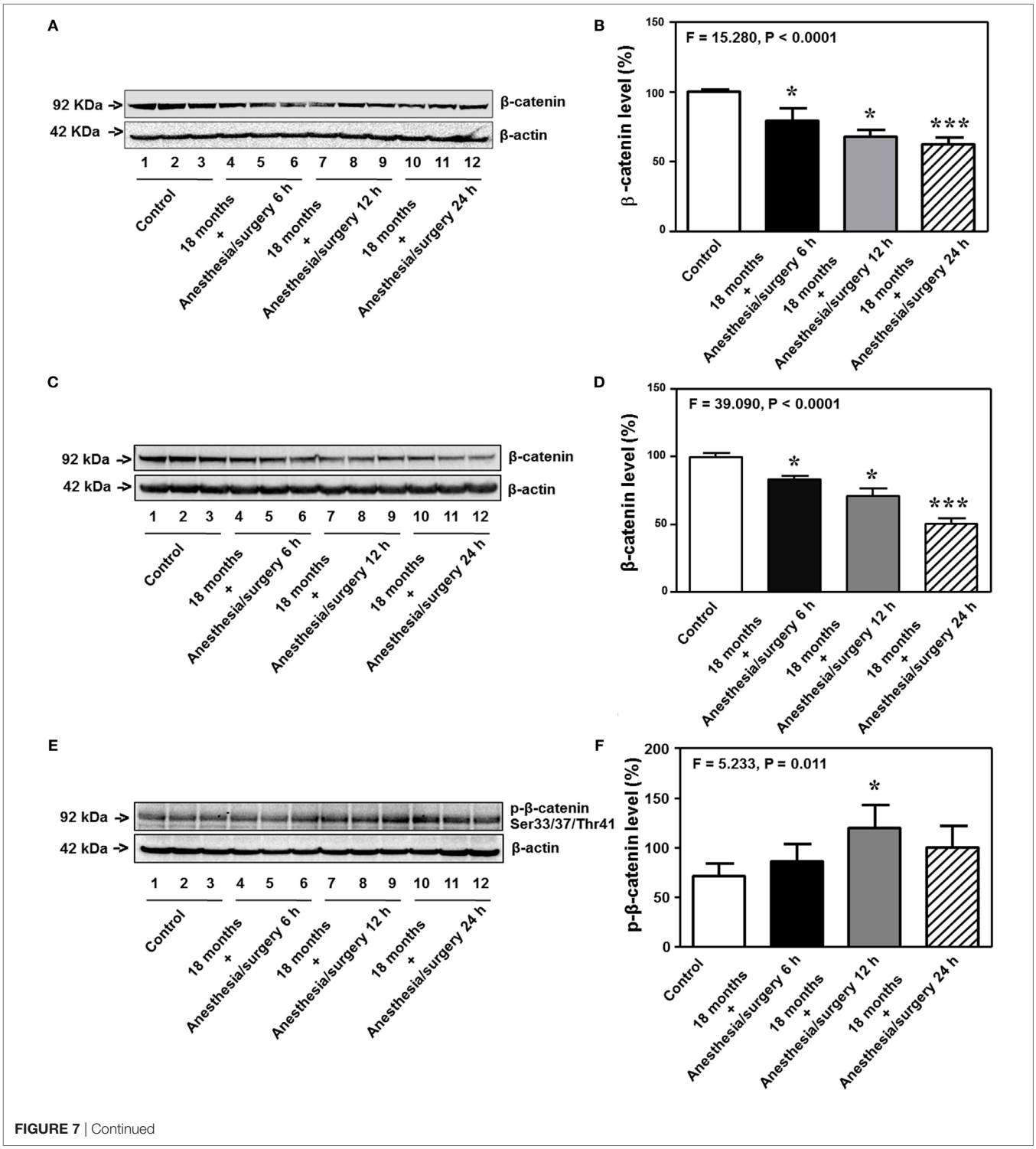

FIGURE 7 | Continued

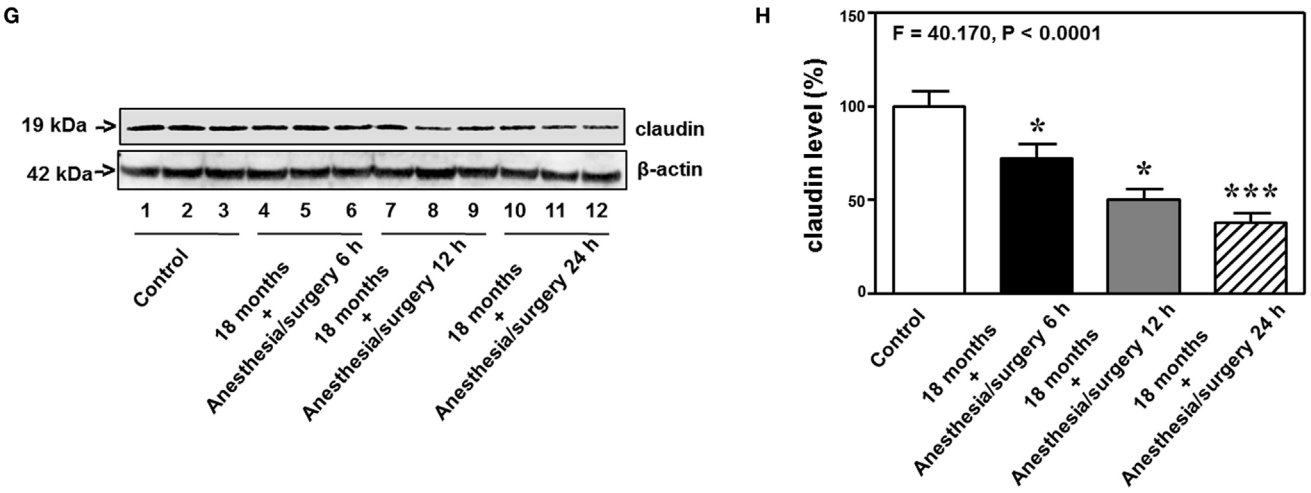

FIGURE 7 | Anesthesia/surgery decreases levels of cell junction proteins. The anesthesia/surgery decreases β-catenin levels in the cortex of 18-month-old mice at 6, 12, and 24 h after the anesthesia/surgery as compared to the control condition **(A,B)**. The anesthesia/surgery decreases β-catenin levels in the hippocampus of 18-month-old mice at 6, 12, and 24 h after the anesthesia/surgery as compared to the control condition **(C,D)**. The anesthesia/surgery increases the phosphorylated β-catenin levels in the hippocampus of 18-month-old mice at 6, 12, and 24 h after the anesthesia/surgery as compared to the control condition **(E,F)**. The anesthesia/surgery reduces the level of claudin in the hippocampus of 18-month-old mice at 6, 12, and 24 h compared to control condition **(G,H)**. *N* = 6 in control or anesthesia/surgery group.

in the hippocampus of the 18-month-old mice. The fifth band on each of the Western blot image represents the level of β-catenin (**Figure 8A**), claudin (**Figure 8C**), occludin (**Figure 8E**), and ZO-1 (**Figure 8G**) obtained from one 18-month-old IL-6 KO mouse. Taken together, these data suggest that treatment of IL-6 antibody or KO of IL-6 gene is able to attenuate the anesthesia/surgery-induced reduction in the levels of cell junction proteins.

DISCUSSION

Anesthesia and surgery (anesthesia/surgery) in rodents have been shown to induce cognitive impairment [(1–10, 33), reviewed in Ref. (11)]. However, the underlying mechanisms still remain largely to be determined. Given the fact that BBB dysfunction could contribute to cognitive impairment [(18, 46–48), reviewed in Ref. (17, 20)], we set out to investigate whether the anesthesia/surgery was able to induce BBB dysfunction, e.g., increase in BBB permeability and cognitive impairment in mice.

Dextran-tracer injection has been used to reveal BBB formation and function (15). We, therefore, employed dextran to assess the effects of the anesthesia/surgery on BBB function in our studies. The anesthesia/surgery induced BBB dysfunction as evidenced by the findings that the anesthesia/surgery increased the BBB permeability to 10-kDa dextran in the brain tissues of mice (**Figures 2A,B**). Moreover, there was greater BBB permeability to the 10-kDa dextran in the brain tissues of 18-month-old mice than that in the brain tissues of 9-month-old mice following the anesthesia/surgery: row 3 versus row 4 in **Figure 2A**; and gray bar versus net bar in **Figure 2B**. Finally, IL-6 antibody (**Figures 2A,B**) and knockout of IL-6 gene (**Figure 2D**) attenuated the anesthesia/surgery-induced increase in BBB permeability to 10-kDa dextran. The anesthesia/surgery may not induce the increase in BBB permeability to larger molecules, e.g., 70-kDa dextran. Finally, the anesthesia/surgery induced an age-associated increase in blood IL-6 levels (**Figure 4**). Collectively, these data support the hypothesis that the anesthesia/surgery induces an age-associated and IL-6 dependent increase in BBB permeability in mice.

Consistent with these data, we were able to show that the anesthesia/surgery induced cognitive impairment in the 18, but not 9, month-old mice (**Figures 5** and **6**). The data suggest that the anesthesia/surgery might cause an age-associated BBB dysfunction, leading to the age-associated cognitive impairment in rodents, pending on further investigation.

Neuroinflammation has been suggested to contribute to postoperative delirium [reviewed in Ref. (49)] and postoperative cognitive dysfunction [reviewed in Ref. (17)]. Specifically, IL-6 has been reported to be associated with learning and memory impairment in animals (50–52), cognitive dysfunction (53), mild cognitive impairment (54), and delirium in patients (55). In the current studies, we found that the anesthesia/surgery-induced an age-associated increase in blood IL-6 level (**Figure 4**); as well as an age-associated and IL-6-dependent BBB dysfunction (**Figures 2** and **3**). These findings further support the role of IL-6 in the neurotoxicity associated with anesthesia and surgery; and suggest that IL-6 may contribute to postoperative delirium and postoperative cognitive dysfunction *via* the impairment of BBB function. Finally, these results postulate the hypothesis that the accumulated IL-6, induced by the anesthesia and/or surgery, could attack the BBB, leading to BBB dysfunction.

The human IL-6 protein has 184 amino acids plus a 28-amino-acid-hydrophobic signal sequence (56). Blood IL-6 can be generated by circulating myeloid or lymphoid cells, or can be released from gut, liver, wound, muscle, and other local tissues [reviewed in Ref. (57)]. Specifically, IL-6 is produced in

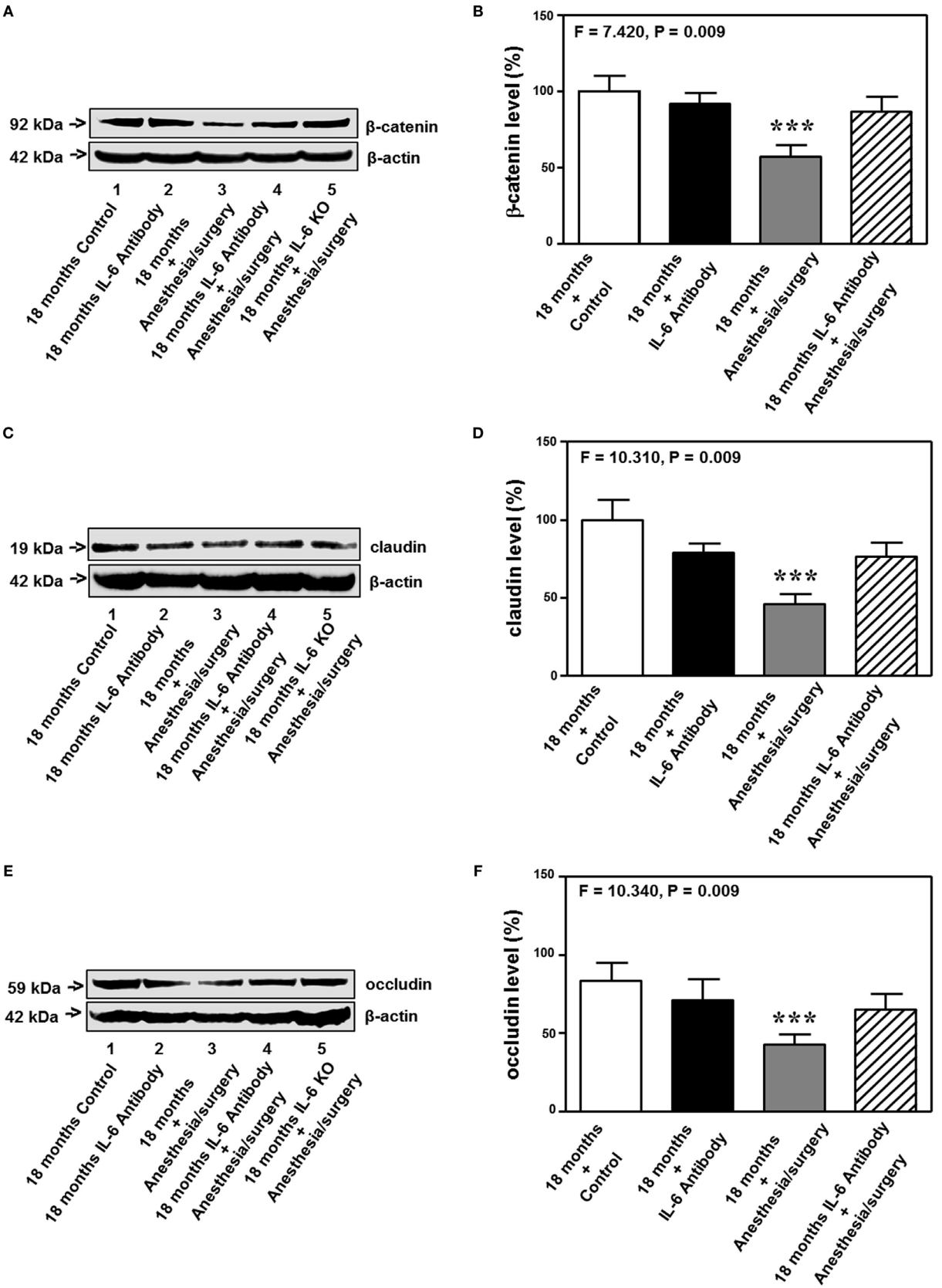

FIGURE 8 | Continued

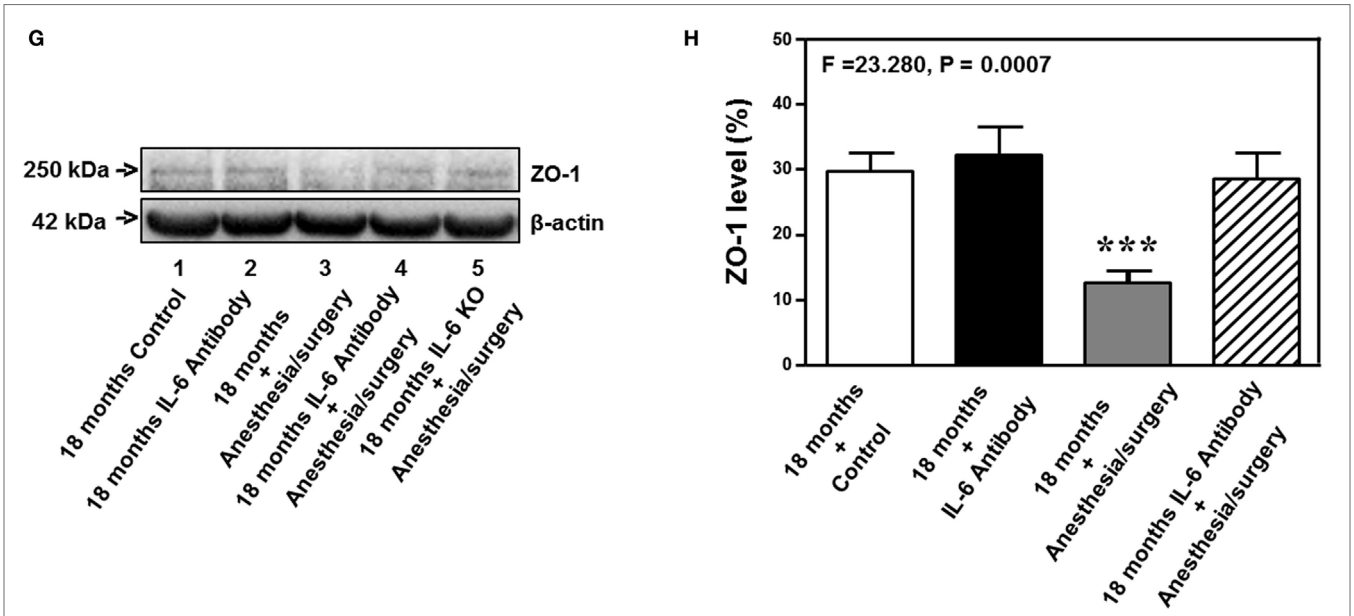

FIGURE 8 | IL-6 antibody attenuates the anesthesia/surgery-induced reduction in cell junction proteins. The anesthesia/surgery reduces protein levels of β-catenin **(A,B)**, claudin **(C,D)**, occuludin **(E,F)**, and ZO-1 **(G,H)** in the hippocampus of 18-month-old mice as compared to control condition at 24 h after the anesthesia/surgery. Treatment of IL-6 attenuates these reductions in the levels of β-catenin **(A,B)**, claudin **(C,D)**, occuludin **(E,F)**, and ZO-1 **(G,H)** induced by the anesthesia/surgery. The knockout of IL-6 gene [lanes 5 in **(A,C,E,G)**] also attenuates the anesthesia/surgery-induced reduction in the levels of β-catenin **(A)**, claudin **(C)**, occludin **(E)**, and ZO-1 **(G)**. IL, interleukin. *N* = 6 in each group except IL-6 gene knockout group of **(A,C,E,G)**.

peripheral blood leukocytes, spleen, liver, kidney, and intestine (58, 59) and the induction of IL-6 generation is in nearly every human tissues and cells [reviewed in Ref. (57)]. A recent study showed that blood monocyte was able to generate IL-6 (60). The data from our current studies have established a system and shown that the anesthesia/surgery can increase blood IL-6 levels and may induce an IL-6-dependent increase in BBB permeability. However, the underlying mechanisms of such findings remain largely unknown. The future studies should assess whether injection of IL-6 is able to increase the BBB permeability in IL-6 KO mice. The future studies should also test a hypothesis that the anesthesia/surgery-induced increase in IL-6 may regulate junction protein levels, BBB permeability, and cognition in mice *via* cellular signal transduction, e.g., Wnt signaling pathway.

Anesthesia/surgery did not increase the BBB permeability of 70-kDa dextran (**Figure 2E**). The exact reason why the anesthesia/surgery increased the BBB permeability to 10-kDa dextran, but not 70-kDa dextran, is not known at the present time. The molecular weights of 70-kDa dextran are larger than that of 10-kDa dextran, it is conceivable that the anesthesia/surgery might only increase BBB permeability to small molecules (e.g., dextran, 10 kDa), but not large molecules (e.g., dextran, 70 kDa).

There are other studies that also demonstrate that anesthesia and surgery induce BBB dysfunction. Dittmar et al. showed that anesthetic isoflurane, administered after hypoxia, was able to induce apoptosis of endothelial cells *in vitro* (61). These findings suggest that isoflurane might damage BBB (61). Acharya et al. reported that anesthetic sevoflurane, but not isoflurane, was able to induce an aging-linked BBB compromise in rats, potentially leading to postoperative cognitive decline and later dementia (62). However, isoflurane was also reported to lead to hippocampal BBB compromise through changes in ultrastructure and occludin tight junction protein expression in aged rats (63). Finally, surgery (splenectomy) under anesthesia (2–3% isoflurane) was able to increase the BBB permeability measured with IgG immunohistochemistry in aged (22- to 23-month-old) rats (64). Consistent with these findings, the data from the current studies demonstrated that the surgery plus anesthesia (1.4% isoflurane up to 2 h) increased the BBB permeability to 10-kDa dextran. However, the data from the current studies further showed that minor surgery (opening and closing of abdominal cavity) under the anesthesia could induce an age-associated BBB dysfunction in mice (**Figures 2** and **3**). More importantly, the results from the current studies suggest that the anesthesia/surgery was able to selectively increase the BBB permeability to 10-kDa dextran, but not to 70-kDa dextran (**Figure 2E**). Finally, the findings from the current studies suggest that the anesthesia/surgery-induced increase in BBB permeability was dependent on IL-6 (**Figures 2D** and **3**). The future studies will use the established system (the anesthesia/surgery-induced BBB permeability) to study whether BBB dysfunction contributes to postoperative cognitive dysfunction and postoperative delirium.

We found that the anesthesia/surgery was able to reduce the levels of tight junction protein claudin, occludin, and ZO-1 (**Figures 7** and **8**), but not adherent junction protein VE-cadherin, E-cadherin, and p120-catenin (Figure S1 in Supplementary Material). These data suggest that the anesthesia/surgery may selectively impair tight junction, but not adherent junction,

leading to the increase in BBB permeability to small molecule, e.g., 10-kDa dextran, but not big molecule, e.g., 70-kDa dextran.

Interestingly, the anesthesia/surgery decreased level of β-catenin. β-catenin exists in three locations: cell membrane, cytosol, and nucleus (65, 66). The cell membrane β-catenin is one of the adherent junction proteins. However, the findings that the anesthesia/surgery increased the levels of phosphorylated β-catenin (Ser33/37/Thr41) suggest that the anesthesia/surgery may specifically decreased the levels of cytosol β-catenin, because the antibody (92 kDa, Cat: #9561, 1:1,000 dilution, Cell signaling, Danvers, MA, USA) we used in the current experiment specifically detected the phosphorylated β-catenin (Ser33/37/Thr41) levels in the cytosol (44). Nevertheless, it is still possible that the anesthesia/surgery could also regulate cell membrane and nucleus β-catenin. The future studies should specifically assess whether the anesthesia/surgery can also change the levels of cell membrane and nucleus β-catenin.

Taken together, the data obtained from the current studies suggest a hypothesized pathway that the anesthesia/surgery can increase plasma IL-6 levels, which regulate the metabolism of cytosol β-catenin, leading to reductions in the levels of tight junction proteins and increase in BBB permeability to small molecule. Consequently, the BBB dysfunction leads to cognitive impairment.

Recent studies have shown that CX3CR1+ monocytes can regulate learning and learning-dependent dendritic spine remodeling *via* TNF-α following infection with poly (I:C), a synthetic analog of double-stranded RNA (60). These data suggest that innate immune-cell activation may contribute to cognitive impairment. Thus, our future studies should include the investigation of the role of CX3CR1+ monocytes on the anesthesia/surgery-induced changes in BBB permeability, levels of junction protein, and cognition in mice.

There are several limitations of the current studies. First, we only assessed the effects of the anesthesia/surgery on BBB permeability in cortex of the mice. The effects of the anesthesia/surgery on BBB permeability in different brain regions (e.g., hippocampus) of the mice could be different. However, the outcomes from the current studies have established a system, which would be used to study the effects of anesthesia and/or surgery on BBB permeability in other brain regions. Second, our present study cannot define cellular sources and targets of the plasma IL-6. What cells release IL-6 and how this affects cells in the neurovascular unit warrant further investigation. Third, we did not use littermate controls for IL-6 KO mice throughout the experiments. We will use the littermate controls for IL-6 KO mice in the future investigation. Finally, we only assessed the effects of the anesthesia/surgery on BBB permeability in cortex to establish a system in the current studies. In the future investigations, we will use the established system to determine the effects of the anesthesia/surgery on BBB permeability in other brain regions (e.g., hippocampus) and the potential association with the observed behavioral changes.

In conclusion, the current studies mainly established a system in adult and older mice to study the effects of abdominal surgery under isoflurane anesthesia (anesthesia/surgery) on BBB permeability and behavioral changes. The data only suggest that the anesthesia/surgery may increase the BBB permeability to 10-kDa dextran, but not 70-kDa dextran, in the cortex of mice. This anesthesia/surgery-induced increase in the BBB permeability to dextran could be dependent on IL-6 and might be greater in older mice (e.g., 18-month-old mice). Moreover, the anesthesia/surgery induced an age-associated cognitive impairment in the mice. Given the fact that BBB dysfunction is associated with cognitive impairment, the results from the current studies suggest that the anesthesia/surgery would induce postoperative delirium and cognitive impairment by damaging BBB function, pending further investigation. These findings would promote further studies of the BBB-associated underlying mechanisms of postoperative delirium and postoperative cognitive dysfunction.

ETHICS STATEMENT

All experiments were performed in accordance with the National Institutes of Health guidelines and regulations. The animal protocol was approved by the Massachusetts General Hospital (Boston, MA, USA) Standing Committee on the Use of Animals in Research and Teaching.

AUTHOR CONTRIBUTIONS

ZX, SY, CG, GY, YS, YZ, XF, and EL conceived and designed the project. SY, CG, EM, EE, YD, and YZ performed all the experiments and prepared the figures. ZX, SY, and CG wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00902/full#supplementary-material.

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The Effect of Propofol vs. Isoflurane Anesthesia on Postoperative Changes in Cerebrospinal Fluid Cytokine Levels: Results from a Randomized Trial

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Introduction: Aside from direct effects on neurotransmission, inhaled and intravenous anesthetics have immunomodulatory properties. *In vitro* and mouse model studies suggest that propofol inhibits, while isoflurane increases, neuroinflammation. If these findings translate to humans, they could be clinically important since neuroinflammation has detrimental effects on neurocognitive function in numerous disease states.

Materials and methods: To examine whether propofol and isoflurane differentially modulate neuroinflammation in humans, cytokines were measured in a secondary analysis of cerebrospinal fluid (CSF) samples from patients prospectively randomized to receive anesthetic maintenance with propofol vs. isoflurane (registered with http://www.clinicaltrials.gov, identifier NCT01640275). We measured CSF levels of EGF, eotaxin, G-CSF, GM-CSF, IFN-α2, IL-1RA, IL-6, IL-7, IL-8, IL-10, IP-10, MCP-1, MIP-1α, MIP-1β, and TNF-α before and 24 h after intracranial surgery in these study patients.

Results: After Bonferroni correction for multiple comparisons, we found significant increases from before to 24 h after surgery in G-CSF, IL-10, IL-1RA, IL-6, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, and TNF-α. However, we found no difference in cytokine levels at baseline or 24 h after surgery between propofol- (n = 19) and isoflurane-treated (n = 21) patients ($p > 0.05$ for all comparisons). Increases in CSF IL-6, IL-8, IP-10, and MCP-1 levels directly correlated with each other and with postoperative CSF elevations in tau, a neural injury biomarker. We observed CSF cytokine increases up to 10-fold higher after intracranial surgery than previously reported after other types of surgery.

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Discussion: These data clarify the magnitude of neuroinflammation after intracranial surgery, and raise the possibility that a coordinated neuroinflammatory response may play a role in neural injury after surgery.

Keywords: anesthesia, cerebrospinal fluid, cytokine, inflammation, isoflurane, propofol, surgery, neuroinflammation

INTRODUCTION

Many intravenous and inhaled anesthetics modulate immunologic and inflammatory function by acting at multiple receptors and ion channels on leukocytes (1–3). Mouse models and *in vitro* studies have shown that propofol has anti-inflammatory effects (4, 5) while isoflurane has pro-inflammatory effects (6). However, studies comparing the impact of propofol vs. inhaled anesthetics (such as isoflurane) on human postoperative serum inflammatory markers have produced mixed results (7–9). Several studies have also show an increase in cerebrospinal fluid (CSF) cytokine levels after surgery. For example, CSF IL-6 levels increased from before to postoperative day (POD) 1 after hip and knee replacements, aortic valve replacement surgeries, esophageal carcinoma resections, and dural leak repairs (10–14). In 11 patients undergoing endoscopic dural leak repairs, CSF IL-6, IL-10, and TNF-α levels were increased on POD1 and POD2, and sevoflurane-treated patients had a greater increase in CSF IL-6 levels than propofol-treated patients (14). However, anesthetic type was not randomized in this small study, so these results could also be explained by underlying differences between the propofol- vs. sevoflurane-treated patients (14). To the best of our knowledge, no randomized controlled study has compared the impact of different anesthetics or intracranial surgery on postoperative CSF cytokine responses. If anesthetics have inflammatory-modulating properties in humans, this could be clinically significant because many human neurologic and neurocognitive disorders are thought to involve brain inflammation [e.g., multiple sclerosis (MS) (15), traumatic brain injury (TBI) (16), Alzheimer's disease (AD) (17), human immunodeficiency virus (HIV)-associated neurocognitive dysfunction (18), and postoperative delirium and cognitive dysfunction (19)]. Many patients with these disorders also undergo anesthesia and surgery each year. Thus, understanding the effects of different anesthetics on human postoperative neuroinflammation is an important goal in perioperative medicine.

To evaluate whether isoflurane treatment is associated with a greater postoperative neuroinflammatory response than propofol treatment, we measured CSF cytokine levels before and 24 h after intracranial surgical procedures. CSF samples were obtained from a previously reported trial (20), which was designed to measure changes in CSF AD biomarkers in patients prospectively randomized to receive propofol vs. isoflurane for anesthetic maintenance.

PATIENTS AND METHODS

Patients

After approval by the Duke University Institutional Review Board, intracranial surgery patients whose case posting included lumbar CSF drain placement were enrolled in the Markers of AD after Propofol vs. Isoflurane Anesthesia (MAD-PIA) trial (20). MAD-PIA was a prospective randomized trial registered with http://www.clinicaltrials.gov (NCT01640275) on June 20, 2012 by Miles Berger, the study PI. Patients were excluded if they were (1) unable to provide informed consent, (2) <18 years old, (3) pregnant, (4) imprisoned, or (5) had a personal or family history of malignant hyperthermia or other medical contraindication to receiving propofol or isoflurane. The primary aim of the parent study was to evaluate the effect of anesthetic type on CSF AD biomarkers, such as amyloid beta, tau, and phosphorylated tau (20). For this study, additional CSF sample aliquots collected during the MAD-PIA trial were used to evaluate the effect of anesthetic type on postoperative CSF cytokine increases. Cognitive impairment (clinical AD, mild cognitive impairment, or other cognitive impairment) was not an exclusion criterion, although patients had to be able to give informed consent. We did not exclude intracranial surgical cases with neuromonitoring (such as brainstem auditory evoked responses).

To determine the impact of surgical procedure type on neuroinflammation, surgical cases were divided into three types (**Table 2**): 1, peripheral neurosurgery without deep intracranial work (e.g., retromastoid craniectomy for trigeminal nerve decompressions, or CSF leak repairs); 2, deep intracranial surgery (e.g., cerebellopontine angle tumor or acoustic neuroma resections); or 3, miscellaneous cases (e.g., cortical surface meningioma resections).

Study Protocol

Patients were randomized to receive either propofol or isoflurane for anesthetic maintenance; however, all patients received propofol for anesthesia induction. Depending on the patient's randomization assignment, anesthesiologists (and anesthesia residents and nurse anesthetists) were instructed to titrate propofol or isoflurane dosage to maintain a bispectral index (BIS) value of 40–60. There were no protocol restrictions regarding the use of other anesthetic drugs, such as opioids, paralytics, or steroids. After anesthetic induction but before surgical incision, a member of the surgery team inserted a subdural Silastic® catheter at the L4–L5 or L5–S1 interspace, and then connected it to an external CSF drain (AccuDrain INS-8400; Integra Neurosciences, Plainsboro, NJ, USA).

CSF Sampling

Fresh CSF samples (10 ml) were obtained from the lumbar drain at the time of drain placement (0 h) and 24 h later, and placed in a 15 ml conical tube (VWR; Radnor, PA, USA) on ice. The CSF samples were divided into 1 ml aliquots in pre-chilled Sarstedt 1.5 ml polypropylene microcentrifuge tubes (VWR; Radnor, PA, USA) with low-binding 1,000 µl pipette tips (Genesee; San Diego, CA, USA) and then stored at −80°C.

CSF Cytokine Measurement

A wide variety of molecules and cells are involved in various human inflammatory disease states (21–24). Since there were little previously published data on which inflammatory molecules may be involved in the human brain's response to intracranial surgery, we assessed a broad spectrum of inflammatory markers using the Millipore 15-plex HCYTOMAG-60K plate (Millipore; Billerica, MA, USA), including EGF, eotaxin, G-CSF, GM-CSF, IFN-α2, IL-10, IL-1RA, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, and TNF-α. Cytokine assays were performed at the Immune Reconstitution Core Laboratory at the Duke Human Vaccine Institute. CSF samples were diluted 1:40 and then assayed in duplicate using the HCYTOMAG-60K panel. The assay was performed according to the manufacturer's recommended protocol and data were obtained (to the 100th digit place) using a Bio-Plex 200 array reader (Bio-Rad; Hercules, CA, USA). Data were analyzed using Bio-Plex manager software (Bio-Rad; Hercules, CA, USA).

Sample Size/Power Analysis

This study aimed to test the hypothesis that propofol and isoflurane have differential effects on postoperative human neuroinflammation. To the best of our knowledge, no prior randomized human study has measured CSF cytokine levels before and after surgery in patients who received propofol vs. isoflurane. Thus, we judged there to be insufficient preliminary data to perform a formal *a priori* sample size calculation. To estimate the potential effect size of anesthetic type upon human postoperative CSF cytokine increases after intracranial surgery, we measured cytokine levels in CSF samples from patients in the MAD-PIA study (20), who were randomized to receive propofol ($n = 19$) vs. isoflurane ($n = 21$) for anesthetic maintenance.

Statistical Analysis

Baseline and intraoperative differences between the propofol and isoflurane treatment groups were evaluated with *t*-tests for continuous variables and chi-square tests for categorical variables in **Table 1**. Changes in cytokine levels from before to 24 h after surgery were evaluated with Wilcoxon signed rank tests (**Table 3**). To account for analysis of multiple cytokines, we used the Bonferroni method to adjust *p* values [**Table 3**; (25)]. Differences in cytokine levels between isoflurane- vs. propofol-treated patients were compared with Wilcoxon ranked sum tests, at both the baseline and 24 h time points (**Figure 2**). Spearman correlation coefficients were used to examine the correlation between changes among CSF cytokines over time and between CSF cytokines and CSF tau levels, and a Bonferroni correction for multiple comparisons was performed (**Figure 3**). A Wilcoxon ranked sum test was used to compare 24 h cytokine levels between patients discharged on POD1 vs. after POD1. For all analyses, significance was set at $\alpha = 0.05$ after multiple comparison correction. Stata 15 SE (Statacorp, College Station, TX, USA) was used for all statistical analyses.

RESULTS

Study enrollment is depicted in **Figure 1**. Baseline and intraoperative characteristics of the study patients who had samples obtained at 0 and 24 h are presented in **Table 1**. None of the patients had a diagnosed neurodegenerative disease or MCI. The isoflurane group was older, received slightly less hydromorphone than the propofol group, and had slightly higher intraoperative bispectral index readings; otherwise, the two groups were generally well balanced. There were more females than males in both groups, but the difference between the groups was not significant. The propofol-treated patients received significantly higher intraoperative propofol dosage, as expected since they were randomized to receive propofol for anesthetic maintenance. There was no significant difference in the surgery type distribution between anesthetic groups (**Tables 1** and **2**).

TABLE 1 | Baseline and intraoperative characteristics for the propofol and isoflurane groups.

	Propofol group ($n = 19$)	Isoflurane group ($n = 21$)	
	Mean (SE) or %	Mean (SE) or %	*p*-Value*
Baseline patient characteristics			
Age	50.2 (3.4)	59.9 (2.6)	0.034
Sex (male/female)	5.3/94.7%	23.876.2%	0.089
Weight (kg)	79.0 (4.9)	82.3 (3.7)	0.465
Body mass index	29.5 (2.2)	29.0 (1.1)	0.957
Intraoperative pharmacologic variables			
Propofol infusion duration (min)	261 (21)	–	
Average propofol drip rate (μg/kg/min)	104.5 (3.7)	–	
Propofol total dosage (mg)	2,114.4 (186.6)	164.1 (27.3)	<0.001
Case length (min)	315 (22)	360 (21)	0.167
Anesthetic duration (min)	311 (22)	361 (23)	0.167
Average MAC[a]	–	0.5 (0)	
Average MAC (h)[a]		2.6 (0.1)	
Total remifentanil dose (mg)	4.2 (0.6)	3.8 (0.5)	0.946
Total fentanyl dose (μg)	258 (20)	252 (11)	0.685
Total hydromorphone dose (mg)	0.9 (0.2)	0.5 (0.1)	0.041
Total dexmedetomidine dose (μg)	7.3 (4.5)	5.6 (1.6)	0.261
Total dexamethasone dose (mg)	13.4 (2.6)	16.9 (2.3)	0.291
Total midazolam dose (mg)	1.4 (0.2)	1.5 (0.2)	0.745
Total clonidine dose (μg)	0 (0)	1.4 (1.4)	0.797
Intraoperative physiologic variables			
Heart rate[a]	70 (3)	68 (2)	0.583
Invasive arterial blood pressure[a]	83 (2)	80 (1)	0.085
Temperature[a]	35.8 (0.3)	35.9 (0.2)	0.698
Pulse oximetry[a]	99 (1)	99 (1)	0.692
Bispectral index[a]	41 (2)	47 (7)	0.031
Surgery type[b]			
1	10	9	
2	7	5	0.2153
3	2	7	

**p Values are from Wilcoxon rank sum tests for continuous variables and chi-square test for categorical variables.*

[a]The propofol treatment group was missing data (number of missing patients) for minute to minute heart rate (2), invasive arterial blood pressure (3), case temperature (10), pulse oximetry (2), and bispectral index (11). The isoflurane treatment group was missing data for average minute to minute MAC (2), MAC-hours (2), heart rate (1), invasive arterial blood pressure (2), case temperature (5), pulse oximetry (1), and bispectral index (5). Gross inspection of the q15 minute vital signs from the intraoperative anesthetic records from these cases showed that the missing heart rate, blood pressure, temperature (except for three cases in which temperature was not recorded), and pulse oximetry values were within 1 SD of the means reported above.

*[b]For surgical type breakdown see **Table 2**.*

TABLE 2 | Surgery types.

Surgery type		Propofol group (n = 19)	Isoflurane group (n = 21)
1	Cerebrospinal rhinorrhea repair	2	1
	Retromastoid craniectomy	8	8
2	Acoustic neuroma resection	2	1
	Craniectomy to cerebellopontine angle	5	4
3	Craniopharyngioma resection	0	1
	Intracranial hemangioma resection	1	0
	Olfactory groove meningioma resection	0	1
	Meningioma—posterior fossa resection	0	1
	Meningioma—supratentorial resection	1	1
	Orbital lesion	0	1
	Pituitary adenoma resection	0	1
	Trigeminal schwannoma resection	0	1

TABLE 3 | Cerebrospinal fluid cytokine levels before and 24 h after surgery (n = 40).

Biomarker	Mean pg/ml (SE) at 0 h	Mean pg/ml (SE) at 24 h	Raw p value	Bonferroni corrected p value
EGF	5.80 (0.73)	6.98 (0.96)	0.3387	>0.99
Eotaxin	9.70 (3.18)	12.17 (3.59)	0.1344	>0.99
G-CSF	20.77 (6.62)	2,170.23 (455.64)	<0.00005	<0.001
GM-CSF	2.75 (0.43)	7.72 (2.95)	0.0942	>0.99
IFN-α2	12.65 (0.81)	16.03 (1.49)	0.2732	>0.99
IL-10	1.57 (0)	133.31 (45.74)	<0.00005	<0.001
IL-1RA	11.79 (7.26)	342.97 (181.44)	0.0001	0.002
IL-6	1.81 (0.16)	11,543.85 (3149.69)	<0.00005	<0.001
IL-7	1.56 (0.04)	2.47 (0.32)	0.0036	0.054
IL-8	97.35 (22.72)	22,388.83 (6926.77)	<0.00005	<0.001
IP-10	645.57 (112.33)	7,984.34 (1,718.99)	<0.00005	<0.001
MCP-1	766.13 (120.82)	35,689.67 (7,469.17)	<0.00005	<0.001
MIP-1α	1.61 (0)	6.92 (1.33)	<0.00005	<0.001
MIP-1β	2.76 (0.6)	117.68 (40.85)	<0.00005	<0.001
TNF-α	1.60 (0)	4.92 (0.78)	<0.00005	<0.001

Cytokine levels were measured in CSF samples obtained before and 24 h after surgery. Even after Bonferroni correction (25), G-CSF, IL-10, IL-1RA, IL-6, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, and TNF-α each showed a significant increase from before to 24 h after surgery (**Table 3**). However, there were no significant differences between propofol vs. isoflurane-treated patients either before or 24 h after surgery (**Figure 2**).

To determine whether postoperative changes in CSF cytokines were correlated with one another, we calculated spearman correlation coefficients between each two-way cytokine combination (i.e., 120 possible two-way combinations). Roughly 40% (or 46) of these 120 possible two-way cytokine elevations were positively correlated with one another (**Figure 3**). Of these correlations, 18 remained significant after Bonferroni correction (25), consistent with a coordinated neuroinflammatory response. We previously reported a trend toward an inverse correlation between intraoperative dexamethasone dosage and the postoperative increase in the neural injury biomarker tau (20), raising the possibility that a steroid-sensitive inflammatory mechanism may be involved in postoperative CSF tau increases. Thus, we examined whether CSF cytokine increases correlated with postoperative CSF tau increases (20). Postoperative increases in CSF IL-6, IL-8, IP-10, and MCP-1 levels each showed a statistically significant correlation with the postoperative increase in CSF tau levels (**Figure 3**).

Since neuroinflammation is thought to have detrimental effects on cognition and might thus be expected to be associated with prolonged hospital length of stay, we also examined the relationship between postoperative cytokine increase and discharge date. Eight of the 40 total patients were discharged by POD 1. There was no difference in CSF cytokine levels from before to 24 h after surgery in the 8 patients discharged at POD 1 vs. the 32 patients discharged after POD 1 [$p > 0.05$ for each cytokine, after Bonferroni correction (25)].

DISCUSSION

Here, we report that anesthetic type had no significant effect upon CSF cytokine levels after intracranial surgery, yet multiple CSF cytokine levels increased significantly after surgery, and many of these CSF cytokine increases correlated with one another and with CSF tau increases. Our comparison of propofol- and isoflurane-treated patients was limited by slight differences in age, hydromorphone dose, and BIS index (**Table 1**), though we believe that these slight differences between groups are unlikely to have obscured a biologically significant difference in cytokines levels. Our results are similar to those of Beck-Schimmer et al., who recently found no difference in LPS-induced neuroinflammation among rats treated with propofol vs. the inhaled anesthetic sevoflurane (26). However, due to insufficient prior literature comparing the human postoperative increase in CSF cytokines between propofol- vs. isoflurane-treated patients, we were unable to perform an *a priori* sample size calculation for this study. Thus, we cannot exclude the possibility that propofol and isoflurane have differential effects on human postoperative neuroinflammation, which may not have been detected here due to a type II statistical error. Nonetheless, the data presented here allow us to perform a *post hoc* sample size calculation for future studies designed to assess whether these anesthetics have differential effects on postoperative CSF cytokine increases. To have 80% power with an $\alpha = 0.05$ to detect a difference similar in magnitude to that seen here for 24 postoperative vs. baseline CSF cytokine levels in propofol- and isoflurane-treated patients, a future study would need the following sample sizes for each cytokine: 126 for G-CSF; 178 for IL-6; 208 for IL-8; 436 for IP-10; 8,450 for MCP-1; 3,886 for MIP-1α; and 3,422 for TNF-α.

Neuroinflammatory responses in other neurocognitive diseases typically involve changes in the levels of numerous cells and molecules over varying time courses (22–24). Of these, here we measured 15 CSF cytokines; future studies should examine other important cytokines, such as IFN-γ and IL-17. The CSF samples in this study were obtained from patients who had CSF drains. CSF drain placement itself may cause an inflammatory response (27), but we found that much higher CSF cytokine increases than other studies that obtained CSF from an intrathecal drain (11, 14), suggesting that CSF drain placement was not the primary cause of the cytokine increases seen here. Indeed, compared

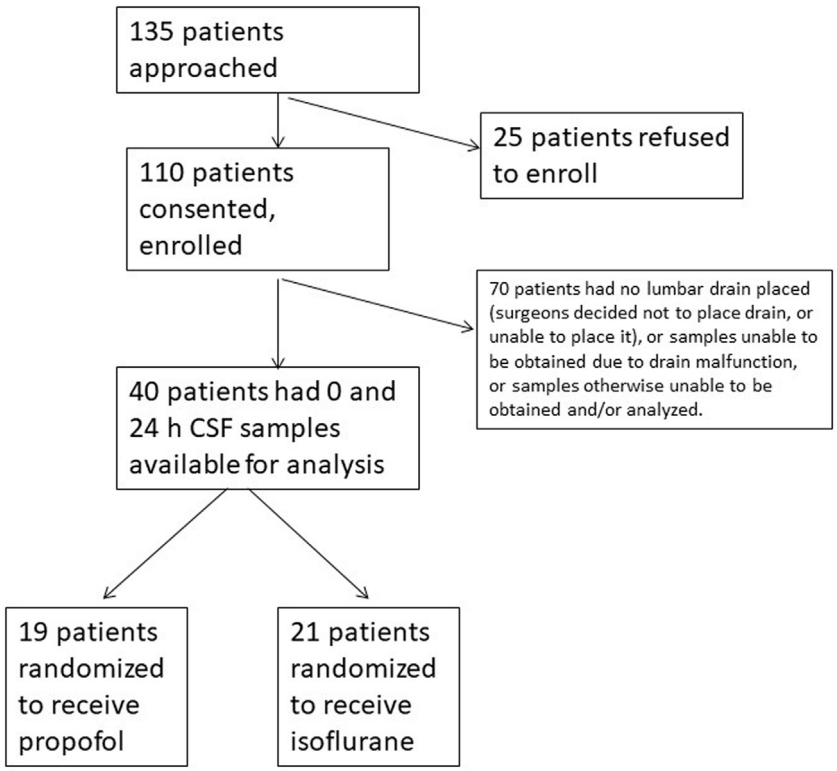


FIGURE 1 | CONSORT diagram/flow chart of the study population. CSF, cerebrospinal fluid.

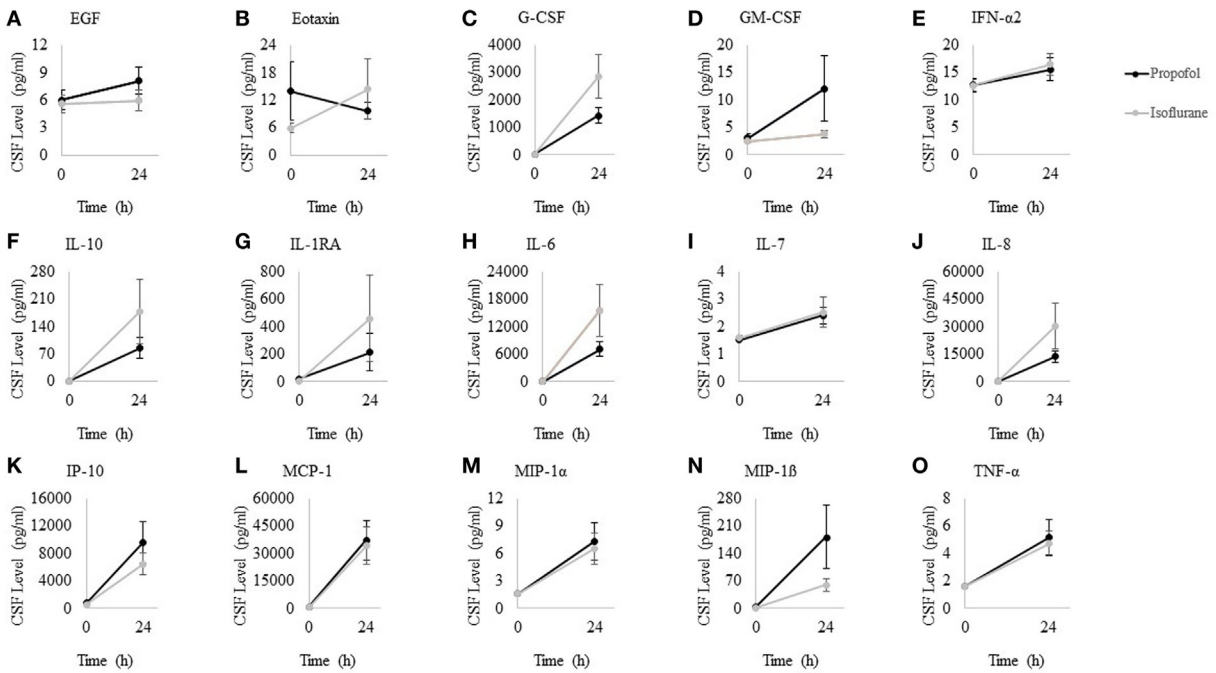
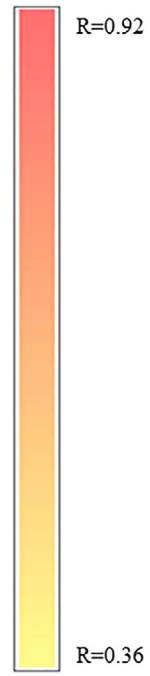


FIGURE 2 | Cerebrospinal fluid (CSF) cytokine levels at 0 and 24 h after surgery for patients randomized to propofol and isoflurane. Wilcoxon rank sum tests showed that was no differences in cytokine levels between propofol and isoflurane-treated patients at either 0 or 24 h ($p > 0.05$ for all, prior to Bonferroni correction). Data points represent anesthetic group means; error bars represent standard error of the mean. **(A)** EGF, **(B)** Eotaxin, **(C)** G-CSF, **(D)** GM-CSF, **(E)** IFN-α2, **(F)** IL-10, **(G)** IL-1RA, **(H)** IL-6, **(I)** IL-7, **(J)** IL-8, **(K)** IP-10, **(L)** MCP-1, **(M)** MIP-1α, **(N)** MIP-1β, and **(O)** TNF-α.

A

	Tau	EGF	Eotaxin	G-CSF	GM-CSF	IFN-α2	IL-10	IL-1RA	IL-6	Il-7	IL-8	IP-10	MCP-1	MIP-1α	MIP-1ß	TNF-α
Tau	1.00															
EGF	-0.07	1.00														
Eotaxin	-0.06	0.37	1.00													
G-CSF	0.20	0.04	0.37	1.00												
GM-CSF	-0.03	0.24	0.50	-0.08	1.00											
IFN-α2	-0.05	0.48	**0.63**	-0.04	**0.64**	1.00										
IL-10	0.34	0.04	0.26	**0.76**	0.10	0.08	1.00									
IL-1RA	-0.05	-0.12	0.23	0.44	0.12	-0.04	0.34	1.00								
IL-6	0.42	-0.05	0.05	**0.88**	-0.21	-0.25	**0.69**	0.38	1.00							
Il-7	-0.18	0.06	0.52	-0.08	**0.64**	0.51	0.02	0.08	-0.13	1.00						
IL-8	0.38	0.04	0.15	0.40	-0.10	-0.15	0.27	0.32	0.55	0.02	1.00					
IP-10	0.42	-0.04	0.20	**0.63**	-0.07	-0.20	0.38	0.35	**0.67**	-0.06	0.46	1.00				
MCP-1	0.57	-0.13	0.12	0.56	-0.06	-0.15	0.39	0.38	**0.72**	0.01	**0.71**	**0.78**	1.00			
MIP-1α	-0.10	0.36	**0.74**	0.27	0.52	**0.67**	0.21	0.14	-0.04	0.46	0.01	0.09	-0.01	1.00		
MIP-1ß	0.19	0.14	0.56	0.56	0.42	0.31	0.46	**0.65**	0.44	0.29	0.36	**0.63**	0.57	0.48	1.00	
TNF-α	-0.09	0.26	**0.72**	0.29	0.48	**0.70**	0.21	0.16	0.01	0.52	-0.05	0.15	0.05	**0.92**	0.46	1.00



B

	Tau	EGF	Eotaxin	G-CSF	GM-CSF	IFN-α2	IL-10	IL-1RA	IL-6	Il-7	IL-8	IP-10	MCP-1	MIP-1α	MIP-1ß	TNF-α
Tau																
EGF	0.71															
Eotaxin	0.74	0.04														
G-CSF	0.28	0.82	0.04													
GM-CSF	0.88	0.20	<0.01	0.65												
IFN-α2	0.81	0.01	**<0.01**	0.84	**<0.01**											
IL-10	0.06	0.84	0.16	**<0.01**	0.60	0.67										
IL-1RA	0.79	0.53	0.22	0.01	0.51	0.81	0.06									
IL-6	0.02	0.79	0.81	**<0.01**	0.26	0.17	**<0.01**	0.04								
Il-7	0.34	0.74	<0.01	0.66	**<0.01**	<0.01	0.92	0.67	0.50							
IL-8	0.04	0.85	0.41	0.03	0.58	0.43	0.14	0.08	<0.01	0.90						
IP-10	0.02	0.83	0.29	**<0.01**	0.72	0.29	0.03	0.06	**<0.01**	0.75	0.01					
MCP-1	<0.01	0.50	0.53	<0.01	0.76	0.42	0.03	0.04	**<0.01**	0.95	**<0.01**	**<0.01**				
MIP-1α	0.61	0.05	**<0.01**	0.15	<0.01	**<0.01**	0.26	0.45	0.83	0.01	0.95	0.61	0.94			
MIP-1ß	0.30	0.46	<0.01	<0.01	0.02	0.09	0.01	**<0.01**	0.01	0.11	0.05	**<0.01**	<0.01	<0.01		
TNF-α	0.62	0.16	**<0.01**	0.11	0.01	**<0.01**	0.25	0.39	0.97	<0.01	0.79	0.41	0.78	**<0.01**	0.01	

FIGURE 3 | Heat map of Spearman correlations between change in cerebrospinal fluid (CSF) cytokine levels from 0 to 24 h after surgery. **(A)** All colored cells are significant at $p < 0.05$. Bolded cells are significant after Bonferroni correction (25). Color bar ranges from yellow ($R = 0.36$ or smallest significant spearman correlation) to red ($R = 0.92$ or largest significant spearman correlation). **(B)** p Values for each comparison in part A [values <0.05 highlighted in blue and values significant after Bonferroni correction are bolded (25)].

to past studies analyzing CSF cytokines after orthopedic, cardiac, gastrointestinal, and CSF leak repair procedures, this study in intracranial surgery patients found approximately a 65× higher level of MCP-1 (11), a 40× higher level of IL-8 (11–13), and a 25× higher level of IL-6 (10, 11, 13, 14) 1 day after surgery. This comparison is limited by the fact that the patients in these prior studies may have differed from the patients in this study in other baseline characteristics (aside from surgery type). Nonetheless,

the massive increases in CSF cytokines reported here raise the possibility that intracranial surgical procedures may be associated with a greater neuroinflammatory response than other types of surgery.

We also found significant correlations among many of these CSF cytokine increases (**Figure 3**), consistent with a coordinated postoperative neuroinflammatory response. Also, CSF IL-6, IL-8, IP-10, and MCP-1 increases correlated with postoperative increases in the CSF neural injury marker tau (**Figure 3**). Pikwer and colleagues recently reported similar though smaller increases in many of these same cytokines in the CSF within 7 h after hysterectomy cases, although they found no tau increase and no correlation between cytokine increases and tau levels (28). The lack of correlation between cytokine increases and tau levels in the Pikwer et al.'s study likely reflects the shorter time course in that study (7 vs. 24 h here), since many of the cytokine trajectories in that paper were still increasing at 7 h, as well as the potentially greater impact of neurosurgery upon the brain (28).

Nonetheless, similar correlations between CSF cytokines and neural injury markers (as well as neurocognitive decline) as those seen here have been reported in both AD (17, 29) and TBI (16). In TBI patients, CSF IL-6 levels of ~10,000 pg/ml have been measured 1 day after trauma (30), and here we found CSF IL-6 levels of ~10,000 pg/ml 24 h after surgery (**Table 3**). Interestingly, CSF IL-10 levels in survivors and non-survivors of TBI at 24 h after trauma were <10 pg/ml (31), while we found IL-10 levels of ~100 pg/ml 24 h after surgery. This further underscores the magnitude of the neuroinflammatory response after intracranial surgery. Taken together, these findings raise the possibility that neuroinflammation after intracranial surgery may contribute to postoperative CSF tau elevation in a similar fashion as that seen in AD and TBI, potentially contributing to neuronal dysfunction after intracranial surgery. Alternatively, CSF cytokine increases may occur secondary to or in response to CSF tau increases (a marker of neuronal damage); future studies will be required to determine the causal relationship between postoperative CSF cytokine and tau increases.

Irrespective of whether CSF cytokine increases cause tau increases or *vice versa*, prior studies have clearly shown that neuroinflammation impairs neurocognitive function at multiple levels ranging from synaptic transmission (32) to functional brain network connectivity (33) to human cognitive performance (34). For example, IL-8 inhibited hippocampal long-term potentiation *in vitro* (32). In addition, intravenous LPS treatment in healthy adults caused altered resting-state functional connectivity within multiple human brain networks (33) and caused increases in IL-6, IL-10, and TNF-α levels in serum and in IL-6 levels within the CSF (35). In fact, neuroinflammation is thought to contribute to both postoperative cognitive dysfunction (POCD) and delirium in older adults (19). Indeed, in a prior study, patients with delirium had higher CSF IL-8 levels (~70 pg/ml) than those without delirium (~40 pg/ml) (36). Furthermore, neuroinflammation has been associated with impaired cognitive performance in multiple human diseases ranging from MS (15) to TBI (16) to AD (17) and HIV (18). However, the role and functional sequelae of the postoperative CSF cytokine increases measured here are unclear. The patients in this trial were not screened for POCD or delirium, as these measures were beyond the scope of this study. Nonetheless, many of these study patients had grossly normal cognition on POD1, and eight were discharged home on POD1. In addition, the discharge summary of 39 of the 40 patients did not contain any indication of delirium, altered mental status, or agitation, although this may reflect a lack of sensitivity in delirium detection/documentation in routine clinical care. There was also no difference in postoperative CSF cytokine increases between the eight patients who were discharged POD1 and those who were discharged after POD1. Together, these results raise the possibility that neuroinflammation alone may be insufficient to cause POCD or delirium, though it may play a role in POCD or delirium in concert with other age-dependent brain changes (37). The magnitude of CSF cytokine increases measured here, together with the known role of neuroinflammation in cognitive impairments in multiple other clinical settings (15–19), suggest that future studies are warranted to determine the cognitive and functional sequelae of postoperative neuroinflammation.

In conclusion, while we found no evidence of an effect of anesthetic on postoperative CSF cytokine increases, there was a highly significant postoperative increase in multiple cytokines. Many of these cytokine increases (i.e., IL-6, IL-8, IP-10, and MCP-1) directly correlated with one another and with CSF tau increases, consistent with a coordinated postoperative neuroinflammatory response. Future studies are needed to further characterize this postoperative neuroinflammatory response and its clinical implications.

ETHICS STATEMENT

This study was carried out with approval from and in accordance with the regulations of the Duke University Medical Center Institutional Review Board. All subjects gave written informed consent in accordance with the Declaration of Helsinki, before any study activities began. The protocol was approved by the Duke University Medical Center Institutional Review Board.

AUTHOR CONTRIBUTIONS

MB conceived of, designed, and oversaw this study, and organized the manuscript. VP and MB wrote the manuscript. JN, MB, AF, DM, DW, and MJ helped enroll patients and obtain samples. MC helped in data extraction and design of statistical analysis, NG helped in design of and performed statistical analysis. LS helped perform and oversee assay measurements. JM, MN, DM, and MJ helped design and oversee the study. All authors made substantial contributions to this work, helped revise the manuscript, approved the final manuscript, and agreed to be held accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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This paper is dedicated to the memory of Dr. William L. Young.

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Neuroprotective Effects of Annexin A1 Tripeptide after Deep Hypothermic Circulatory Arrest in Rats

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Resolution agonists, including lipid mediators and peptides such as annexin A1 (ANXA1), are providing novel approaches to treat inflammatory conditions. Surgical trauma exerts a significant burden on the immune system that can affect and impair multiple organs. Perioperative cerebral injury after cardiac surgery is associated with significant adverse neurological outcomes such as delirium and postoperative cognitive dysfunction. Using a clinically relevant rat model of cardiopulmonary bypass (CPB) with deep hypothermic circulatory arrest (DHCA), we tested the pro-resolving effects of a novel bioactive ANXA1 tripeptide (ANXA1sp) on neuroinflammation and cognition. Male rats underwent 2 h CPB with 1 h DHCA at 18°C, and received vehicle or ANXA1sp followed by timed reperfusion up to postoperative day 7. Immortalized murine microglial cell line BV2 were treated with vehicle or ANXA1sp and subjected to 2 h oxygen–glucose deprivation followed by timed reoxygenation. Microglial activation, cell death, neuroinflammation, and NF-κB activation were assessed in tissue samples and cell cultures. Rats exposed to CPB and DHCA had evident neuroinflammation in various brain areas. However, in ANXA1sp-treated rats, microglial activation and cell death (apoptosis and necrosis) were reduced at 24 h and 7 days after surgery. This was associated with a reduction in key pro-inflammatory cytokines due to inhibition of NF-κB activation in the brain and systemically. Treated rats also had improved neurologic scores and shorter latency in the Morris water maze. In BV2 cells treated with ANXA1sp, similar protective effects were observed including decreased pro-inflammatory cytokines and cell death. Notably, we also found increased expression of ANXA1, which binds to NF-κB p65 and thereby inhibits its transcriptional activity. Our findings provide evidence that treatment with a novel pro-resolving ANXA1 tripeptide is neuroprotective after cardiac surgery in rats by attenuating neuroinflammation and may prevent postoperative neurologic complications.

Keywords: memory, neuroinflammation, NF-κB, postoperative cognitive dysfunction, resolution of inflammation, surgery

INTRODUCTION

Resolution of inflammation was once believed to be a passive process, but we now know that it involves a cascade of coordinated events that is initiated as inflammation begins (1, 2). Defective resolution and non-resolving inflammation contribute to a chronic and maladaptive state that characterizes several diseases ranging from atherosclerosis to rheumatoid arthritis (3). Endogenous mediators, including lipids biosynthesized from omega-3 fatty acids, gases such as carbon monoxide, and certain proteins, promote resolution of inflammation, and restore homeostasis without causing unwanted side effects by optimizing the body's natural chemistry to safely regulate inflammatory molecules (4). Indeed, novel treatment strategies for inflammatory conditions use "resolution agonists" to modulate and enhance these endogenous mediators and signaling pathways (5, 6).

Annexin A1 (ANXA1), a 37-kDa glucocorticoid-regulated protein, is an exemplary resolution agonist that signals through the G protein-coupled receptors FPR2/ALX and FPR1 to regulate calcium influx into the cell (7). ANXA1 also exerts potent anti-inflammatory actions by regulating leukocyte diapedesis, efferocytosis, and pro-inflammatory mediators following infection or injury (8, 9).

Strategies to activate these endogenous inflammation "stop signals" are gaining considerable interest (10). This innovative line of research has led to ANXA1 peptidomimetics, which are designed to boost activation of naturally occurring pro-resolving and anti-inflammatory mechanisms (11). Perretti and colleagues first developed a peptide that is modeled on the first 50 amino acids in the N-terminal portion of ANXA1 (CR-AnxA1$_{2-50}$), and that binds specifically to FPR2/ALX, and exerts key pro-resolving actions in different inflammatory conditions (12–14).

Cardiopulmonary bypass (CPB) with deep hypothermic circulatory arrest (DHCA) is routinely performed during cardiac surgery for repair of thoracic aortic disease or complex congenital cardiac defects. Although this procedure remains necessary to maintain circulation of blood and oxygen while repairing the heart, it contributes to profound perturbations in inflammatory, hemostatic, and oxidative stress pathways, collectively implicated in the pathogenesis of perioperative cerebral injury (15–17). This inflammatory response is specifically activated *via* several pathways: the contact activation by the foreign surface of the CPB circuit, surgical trauma as well as the effect of ischemia–reperfusion (I/R) injury, and endotoxemia (18). Further, its effects have been related to central nervous system injury, including complications like delirium and postoperative cognitive dysfunction (19). Systemic inflammation after both cardiac and non-cardiac surgery can affect the brain *via* neuroinflammatory processes that are amplified by circulating pro-inflammatory cytokines in blood and cerebrospinal fluid (20–23) and localized neuronal impairments (24, 25).

We previously discovered a novel ANXA1 peptidomimetic (ANXA1sp or Ac-QAW) that suppresses human colon cancer growth *via* modulation of NF-κB activation (26). In the current study, we tested the effects of ANXA1sp on postoperative neuroinflammation and cognitive changes in an established rat model of CPB with DHCA and hypothesized that its pro-resolving mechanisms following I/R injury are mediated *via* attenuation of microglial activation.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Duke University Animal Care and Use Committee. All procedures were in accordance with the guidelines of the National Institutes of Health for animal care (Guide for the Care and Use of Laboratory Animals, Health and Human Services, National Institutes of Health Publication No. 86-23, revised 1996). Studies were conducted on adult male Sprague-Dawley rats (age 14–16 weeks; weight 400–450 g; Charles River Laboratories, Wilmington, MA, USA). Rats were housed (two animals per cage) in a 12-h light/dark cycle environment with free access to food and water. Rats were acclimated for at least 1 week before starting any experiment.

Drug Treatments

Annexin A1 biomimetic tripeptide (ANXA1sp or Ac-QAW, Ac = acetyl, MW = 445.47 Da) was synthesized and purified (>98% purity) by GenScript (Piscataway, NJ, USA). The peptide was suspended in 100% DMSO. For *in vivo* experiments, this stock solution was diluted in saline to a final dose of 1 mg/kg ANXA1sp and a concentration of 1% DMSO. For *in vitro* experiments, the ANXA1sp–DMSO stock solution was diluted with culture medium to final concentrations ranging from 5 to 100 μM ANXA1sp. Vehicle control was 1% DMSO in saline for *in vivo* studies, and 1% DMSO in culture medium for *in vitro* studies. ANXA1sp treatment solutions were prepared fresh immediately before use for *in vivo* and *in vitro* experiments.

Short-term Survival Groups (3, 6, and 24 h)

Rats were randomly assigned to six groups (n = 5/group) and terminated for histologic and biochemical analyses at 3, 6, or 24 h after CPB/DHCA. Rats received ANXA1sp (1 mg/kg iv) or vehicle (1% DMSO iv) in 1 mL saline 1 h before CPB and 1 h after reperfusion. Rats in the 24-h survival group were also treated at 6 h after reperfusion. All treatments were administered in a blinded manner.

Long-term Survival Group (Day 7 Post Operation)

Rats were randomly assigned to two groups (n = 10/group) and treated as described above and then daily (ip) up to day 7 post operation. After neurologic and cognitive assessments on day 7 post operation, animals were terminated.

Cardiac Surgery with CPB/DHCA

Fasted rats were anesthetized with isoflurane, intubated, and cannulated for CPB and DHCA without sternotomy, to allow for long-term survival, as previously described (27). Routine physiologic parameters, and pericranial and rectal temperature were continuously monitored. The heparinized CPB circuit consisted of a venous reservoir, a peristaltic pump, a custom-designed membrane oxygenator, and a flow probe. Lung ventilation was stopped for the entire period of CPB/DHCA. Following heparin administration, CPB was initiated at a flow rate of 160–180 mL/

kg/min, which was then decreased as the animals were cooled over 30 min to a target pericranial temperature of 18°C. After reaching 18°C, the rats were subjected to DHCA, which was confirmed by electrocardiographic asystole and absence of any measurable MAP. After 60 min of DHCA, CPB was reinstituted, and rats were rewarmed over 30 min to a pericranial temperature of 34°C. CPB was then terminated, and mechanical ventilation resumed. After 2 h of continuous monitoring, rats were extubated, and recovered in a warmed oxygen-enriched environment with free access to water. Rats in the sham group ($n = 3$/group) were cannulated without exposure to CPB/DHCA; naïve rats were sacrificed under 5% isoflurane.

To harvest the brain, rats were re-anesthetized, intubated, and mechanically ventilated. One sample of brain tissue was immediately fixed in 10% buffered formalin and paraffin-embedded for immunostaining. The remaining brain tissue was frozen in liquid nitrogen and stored at −80°C until further use. Blood samples from each animal were also collected and stored at −80°C until analysis.

Immunostaining of Microglia

Staining was performed on slices (20 μm thick) of the paraffin-embedded brain tissue samples using ionizing calcium-binding adaptor molecule 1 (Iba1) rabbit antibody (Wako Chemicals USA Inc., Richmond, VA, USA). For antigen retrieval, tissue slices were incubated with 10 mM citrate buffer, pH 6.0, for 5 min at 100°C. After the buffer solution cooled to room temperature (RT), slices were washed, and then blocked with 10% normal goat serum for 60 min at RT. Slices were then incubated with primary rabbit anti-Iba1 primary antibody (1:200) overnight at 4°C. After three washes with PBS, the slices were incubated with goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:500, Invitrogen, Carlsbad, CA, USA) for 60 min at RT. Images were captured on a fluorescence microscope (Leica DM IRB, Germany) using a 10×/0.3 PH objective at 1.5-fold magnification. For quantification the total number of Iba1-positive cells was determined in five representative areas of the cerebral cortex (retrosplenial and posterior parietal cortex) and the hippocampus (CA1–CA3 area). Automated imaging and high-content analysis of microglia were done on the Cellomics ArrayScan IV platform and instrument (Thermo Fisher Scientific) using the Target Activation algorithm module optimized for object size, object shape, and fluorescence intensity to identify Iba1 positive cells by soma size (28). Microglial morphology was evaluated using a 4-scale classification method based on (29). Cells were classified based on their overall morphology as (1) round/amoeboid microglia, (2) stout microglia, (3) thicker longer ramifications, and (4) thinner ramifications by an investigator blinded to the experimental groups.

Cell Death Assessment

Apoptosis was determined by terminal deoxynucleotidyl nick-end labeling (TUNEL) per assay manufacturer's protocol (Roche Diagnostics, Indianapolis, IN, USA). Briefly, sections of the paraffin-embedded brain tissue sample (5 μm thick) were deparaffinized using xylene and descending grades of ethanol, and pretreated with microwave radiation (350 W, in 200 mL of 0.1 M Citrate buffer, pH 6.0) for 5 min. Tissue sections were then incubated with terminal deoxynucleotidyl transferase (TdT) for 1.5 h at 37°C and then rinsed with PBS. Slides of five representative areas of the retrosplenial and posterior parietal cortex and CA1 area of the hippocampus were mounted using UltraCruz™Mounting Medium with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative controls were incubated in label solution without TdT. A separate set of sections was stained with acid fuchsin–celestine blue to identify possible necrotic cells. Cell counting was performed in a blinded manner across five representative areas of the cerebral cortex and CA1 areas using a fluorescence microscopy (Leica DM IRB, Germany) with a 20×/0.4 PH objective at 1.5-fold magnification. Data obtained in every field were added together to make a final data count for each slide and expressed as percentage of total cell number within the relevant fields. For *in vitro* cell death assessment, cell culture medium (for necrosis) and cell lysate (for apoptosis) were assayed using the Cell Death Detection ELISA^{PLUS} per manufacturer's protocol to measure cytoplasmic histone-associated DND fragments (momo- and oligonucleosomes) as previously described (30).

Western Blots

Frozen brain samples were homogenized and protein quantified by BCA assay (Thermo Fisher Scientific). Western blotting was performed using SDS-PAGE 4–15% gradient gels (Bio-Rad) with the following antibodies: rabbit polyclonal against phosphor-p65; and ANXA1 (all from Cell Signaling Technology, Danvers, MA, USA). The bands were detected by Super-Signal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA). Band intensities of phosphor-p65 or ANXA1 were normalized with a loading control of β-actin.

Neurologic Evaluation

On day 3 and day 7 post operation, rats underwent standardized functional neurologic testing by an observer blinded to group assignment, using an established neurologic scoring system that evaluates motor deficit (31). Briefly, rats were first placed on a 35 cm × 31.25 cm screen (grid size 0.6 cm × 0.6 cm) that could be rotated from horizontal (0°) to vertical (90°). The length of time that the rat could hold onto the screen after being rotated from 0 to 90° was recorded to a maximum of 15 s (0–3). Rats were then tested for balance on a horizontal wooden rod, and the time lapse before falling off the rod was recorded to a maximum of 30 s (0–3). Finally, rats underwent a prehensile traction test, and the length of time that the rat could cling to a horizontal rope was recorded to a maximum of 5 s (0–3). Animals received a score for each of the three tests. The final score was the sum of the individual test scores, with 0 the best score, and 9 the worst score.

Morris Water Maze

The Morris water maze consisted of a pool of water (27°C), 1.5 m in diameter and 30 cm deep, with a hidden platform submerged 3 cm below the surface in one quadrant, and a computerized video tracking system (EthoVision®; Noldus, Wageningen, The Netherlands) (32). Rats were placed in the water in a dimly lit room with visual clues around the maze. The time to locate the submerged platform (defined as the escape

latency) was measured. From day 3 through day 7 post operation, rats underwent daily testing in the water maze. Four trials were performed each day with an intertrial interval of 10 min. Each trial started in a different quadrant and was limited to 90 s of water exposure. A probe trial was performed on the last day of testing, and the submerged escape platform was removed from the water maze.

BV2 Cell Culture and Hypoxic Exposure

Immortalized murine microglial cell line BV2 were maintained in Dulbecco's modified Eagle's medium (DMEM high glucose) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin in a 37°C humidified incubator with 5% CO_2 (balanced with air). Confluent cultures were passaged by trypsinization. After incubating for 24 h, cells were exposed to ANXA1sp (0, 5, 10, 20, 30, 40, 50, or 100 μM) for 1 h. Cells were then subjected to 2 h oxygen–glucose deprivation (OGD: DMEM no glucose, 85% N_2/10% H_2/5% CO_2) in an OGD chamber (Farma Scientific), followed by reoxygenation for 3, 6, or 24 h in a 37°C growth incubator with 5% CO_2 (balanced with air). Cells treated with 1% DMSO in the medium served as vehicle control. At the end of each time point, cells and culture supernatant were harvested for further analysis.

Cell Viability/MTT Assay

Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetra-zolium bromide) assay per manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, a volume of MTT stock solution (5 mg/mL) equal to one-tenth the original culture volume was added to each culture to be assayed. After incubating for 3 h, cells were centrifuged at 800 *g* for 5 min, and the medium was removed. The formazan crystals were dissolved/solubilized in acidic isopropanol (0.04–0.1 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630–690 nm. Results were presented as cell viability (%) = average O.D. of treatment wells/average O.D. of vehicle-control wells.

NF-κB DNA Binding Activity

Nuclear proteins were extracted from BV2 microglia or cerebral tissues per manufacturer's protocol (Nuclear Extraction Kit, Panomics, Santa Clara, CA, USA). Protein concentration of nuclear extracts was measured using the BCA assay (Thermo Fisher Scientific, Grand Island, NY, USA). NF-κB DNA binding activity was assessed using a quantitative detection kit (Transcription Factor Assay Kit, Cayman Chemical, Ann Arbor, MI, USA). According to the manufacturer's protocol, the 96-well plates were pre-coated with the specific double-stranded DNA sequence that contains the transcription factor NF-κB (p65) response element. Approximately 10 μg nuclear protein was incubated in the coated plate at RT for 1 h while rocking the plate gently at 150 rpm. After washing, NF-κB (p65)-specific primary antibody (1:100 dilution) was added, followed by horseradish peroxidase-labeled secondary antibody (1:100 dilution). The absorbance was read at 450 nm on a microplate reader.

Cytokine Measurement

The concentrations of TNF-α and IL-6 in cell media, plasma, and brain homogenates were measured using rat-specific ELISA kits per manufacturer's protocol (Thermo Fisher Scientific, Grand Island, NY, USA). The plasma was obtained by centrifugation at 2,000 *g* for 10 min at 4°C, and stored at −80°C until use. Brain homogenates were separated by centrifugation at 14,000 *g* for 10 min at 4°C to remove cellular debris. Change in absorbance in every well was detected at 450 nm on a microplate reader. All measurements were performed in triplicate.

Myeloperoxidase (MPO) Measurement

Myeloperoxidase activity in brain tissue, whole cell lysates, and plasma was assessed using ELISA with a rat-specific MPO assay kit per manufacturer's protocol (HK105, HyCult Biotechnology, Uden, The Netherlands).

Confocal Microscopy

After deparaffinization, sections of the brain tissue sample were treated with 10 mM citrate buffer (pH 6.0) for antigen retrieval. After blocking with 10% normal goat serum at RT for 1 h, the sections were incubated with rabbit anti-ANXA1 antibody (1:500) and mouse anti-NF-κB p65 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The sections were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 550-conjugated goat anti-mouse IgG (1:500; Invitrogen, Carlsbad, CA, USA) at RT for 1 h. After washing with PBS, slides of the sections were prepared and mounted using UltraCruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect nuclei.

For *in vitro* confocal microscopy, adherent BV2 cells grown on coverslips were fixed by adding 4% paraformaldehyde to the medium, and incubating for 15 min at RT. After rinsing with PBS, coverslips were permeabilized in freshly prepared 0.1% Triton X-100 and 0.1% sodium citrate for 10 min at RT. After washing and blocking with 10% normal goat serum and 1% BSA for 1 h at RT, coverslips were incubated with rabbit anti-ANXA1 antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-NF-κB p65 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Coverslips were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 555-conjugated goat anti-mouse IgG (1:1,000; Invitrogen, Carlsbad, CA, USA) at RT. Coverslips were mounted using UltraCruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect nuclei. Images were captured on a Leica SP5 confocal microscope (Leica Microsystems, Germany) using a 63×/1.25-0.75 Plan APO oil objective, and the images were analyzed by NIH ImageJ software (version 1.51).

Statistical Analysis

Statistical analysis was performed using Statview Software (version 5, SAS Institute, Cary, NC, USA) and graphs presented with Prism 7 (GraphPad Software, San Diego, CA, USA). Results were expressed as mean ± SD. Morris water maze performance was

compared by repeated measures analysis of variance, with time as the repeated measure and Fisher's least significance difference *post hoc* test. The Mann–Whitney *U* test was used to compare neurologic scores between groups at each recovery interval. Parametric values, including physiologic values, data from ELISA, western blots, as well as numbers of apoptotic and necrotic cells, were compared between groups using the Student's *t*-test. One-way or two-way analysis of variance followed by Tukey's or Sidak's multiple comparison *post hoc* was used as defined in the figure legends. Statistical significance was assumed when $P < 0.05$.

RESULTS

No deaths were reported after CPB/DHCA in this study. Intraoperative physiologic values (MAP, Hct, glucose, pH, $PaCO_2$, PaO_2, HCO_3^-, and pericranial temperature) in rats treated with vehicle or ANXA1sp are summarized in **Table 1**. Intergroup comparisons show no statistical differences, with all values within normal limits.

ANXA1sp Treatment Attenuates Neuroinflammation and Systemic Inflammation after CPB/DHCA

Neuroinflammation is a critical hallmark in several neurocognitive disorders (33). After CPB/DHCA, we found a significant increase in brain levels of key pro-inflammatory cytokines such as TNF-α and MPO (**Figure 1**). TNF-α was elevated 24 h after surgery, with protein levels significantly reduced following ANXA1sp treatment (**Figure 1A**, 12.39 ± 0.11 vs 1.50 ± 0.09, $P < 0.01$). Similarly, MPO levels were lower in ANXA1sp-treated rats at 6 and 24 h compared to controls (**Figure 1B**, 1.47 ± 0.31 vs 0.53 ± 0.14 at 6 h; 0.71 ± 0.24 vs 0.53 ± 0.14 at 24 h, $P < 0.01$). Given the known effects of CPB surgery on the systemic inflammatory response we also measured plasma levels of these pro-inflammatory cytokines and IL-6. Levels of TNF-α, MPO, and IL-6 were elevated after injury, peaking at 6 h and returning toward baseline at 24 h. ANXA1sp-treated rats had significantly blunted systemic inflammation (**Figure 2**). Although statistically significant changes were measured only at 24 h, both 3 and 6 h treated groups had lower levels of plasma TNF-α (**Figure 2A**, 3.63 ± 0.68 vs 0.88 ± 0.17 at 24 h, $P < 0.01$). Systemic levels of MPO were reduced at 6 and 24 h after treatment (**Figure 2B**, 10.12 ± 2.33 vs 4.87 ± 0.30, $P < 0.05$; 6.07 ± 0.31 vs 3.15 ± 0.86, $P < 0.01$), whereas IL-6 was reduced at all time points (**Figure 2C**, 33.20 ± 15.81 vs 11.83 ± 2.33 at 3 h, $P < 0.05$; 58.94 ± 11.79 vs 12.29 ± 10.80 at 6 h, $P < 0.01$; 4.71 ± 1.36 vs 2.27 ± 0.04 at 24 h, $P < 0.05$).

Regulation of Microglial Activation and Cell Death by ANXA1sp after CPB/DHCA

Microglia are resident immune cells in the CNS that have key functions in homeostasis and disease development (34). Changes in microglial morphology are often associated with pathological states. Here we found that CPB/DHCA induced distinct changes in microglial activation both in the hippocampus CA1–CA3 area and retrosplenial and posterior parietal cortex (**Figure 3**). Using a method identified to characterize microglia morphology (29) we found ANXA1sp treatment (1 mg/kg) significantly attenuated microglial activation (**Figures 3A,B**). This was evidenced by reduced numbers of Iba1-positive cells in the hippocampus CA1–CA3 area ($P < 0.01$) and retrosplenial and posterior parietal cortex ($P < 0.01$) at 24 h post reperfusion (**Figure 3A**). This was particular significant for microglial with thicker processes (scale 3, **Figure 3C**) as well as stout microglia in the cortex (scale 2, **Figure 3D**). Overall, CPB/DHCA surgery did not induce significant round/ameboidal microglia (scale 1) at this time point. However, overall number of microglia both in the hippocampus and cerebral cortex were reduced using automated imaging and high-content analysis of soma size (**Figure 3E**).

Finally, ANXA1sp treatment was also associated with a significant reduction in TUNEL-positive cells in the cerebral cortex, but not in the hippocampus, at 24 h after CPB/DHCA (**Figure 4A**). Staining with acid fuchsin–celestine blue also revealed acidophilic neurons and possible necrosis in the hippocampus (**Figure 4B**)

TABLE 1 | Intraoperative physiologic data.

Parameter	Group	Pre-CPB	30 min CPB	60 min DHCA	10 min Reperfusion	End of CPB	120 min P-CPB
MAP (mmHg)	Vehicle	70 (7)	30 (4)	–	72 (25)	88 (11)	88 (6)
	ANXA1sp	66 (7)	29 (4)	–	49 (12)	78 (22)	81 (17)
Temp. (°C)	Vehicle	34.1 (0.8)	19.3 (0.6)	15.0 (0.1)	21.9 (3.0)	33.2 (0.6)	36.0 (0.8)
	ANXA1sp	34.2 (0.3)	19.1 (0.5)	15.0 (0.1)	22.0 (2.3)	33.8 (0.5)	36.4 (0.3)
Glucose (mg/dL)	Vehicle	124 (40)		–		190 (44)	143 (42)
	ANXA1sp	123 (46)		–		170 (36)	116 (38)
PH	Vehicle	7.36 (0.08)	7.46 (0.05)	–	7.57 (0.08)	7.28 (0.05)	7.36 (0.03)
	ANXA1sp	7.38 (0.02)	7.48 (0.09)	–	7.51 (0.15)	7.29 (0.02)	7.40 (0.05)
PO_2 (mmHg)	Vehicle	227 (46)	516 (47)	–	374 (69)	245 (114)	256 (71)
	ANXA1sp	253 (93)	474 (77)	–	257 (211)	170 (138)	247 (126)
PCO_2 (mmHg)	Vehicle	53.2 (13.1)	40.2 (3.8)	–	28.6 (4.6)	55.8 (8.7)	52.0 (4.4)
	ANXA1sp	49.4 (5.1)	38.6 (9.7)	–	31.2 (12.0)	53.0 (5.1)	48.6 (6.1)
HCO_3^- (meq/L)	Vehicle	29.4 (1.9)	24.6 (0.8)	–	25.7 (2.3)	25.7 (2.6)	29.0 (1.0)
	ANXA1sp	29.2 (1.6)	28.3 (1.2)	–	23.5 (1.7)	25.2 (1.9)	29.8 (1.4)
Hct (%)	Vehicle	41.4 (2.3)	25.0 (0.7)	–	25.0 (1.2)	28.0 (0.8)	39.0 (2.9)
	ANXA1sp	41.6 (1.9)	24.2 (0.8)	–	25.2 (0.8)	27.4 (2.6)	38.4 (2.1)

Values in brackets represent SD (– indicates no values due to the bypass machine being on).
CPB, cardiopulmonary bypass; DHCA, deep hypothermic circulatory arrest.

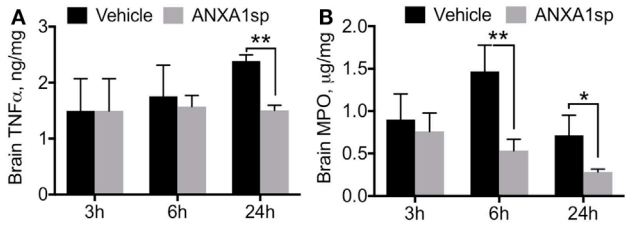

FIGURE 1 | Cytokine levels in brain homogenates after cardiopulmonary bypass/deep hypothermic circulatory arrest by treatment group. ELISA assays revealed time-dependent reductions in brain levels of TNFα **(A)** and myeloperoxidase **(B)** in ANXA1sp-treated rats, with significant effects at 6 and 24 h post reperfusion. Data are presented as mean ± SD (n = 3–5 rats/group). *P < 0.05, **P < 0.01 compared to vehicle controls, analyzed with unpaired t test.

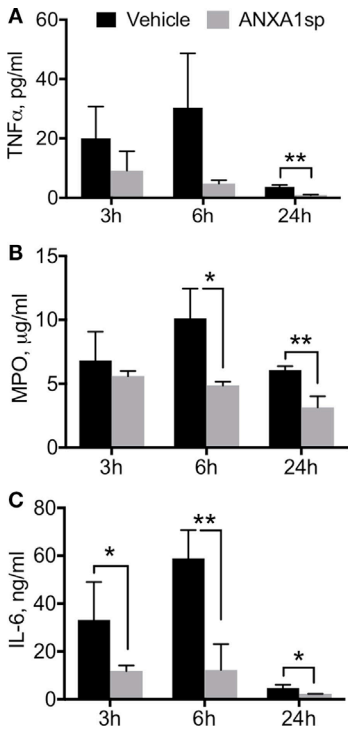

FIGURE 2 | Circulating cytokine levels after cardiopulmonary bypass/deep hypothermic circulatory arrest by treatment group. ELISA assays of plasma levels of TNFα **(A)**, myeloperoxidase (MPO) **(B)**, and IL-6 **(C)** showed that time-dependent effects were noted in TNFα and MPO levels, with significant reduction in ANXA1sp rats by 24 h post reperfusion. IL-6 levels were significantly reduced in ANXA1sp-treated rats as early as 3 h post reperfusion. Data are presented as mean ± SD (n = 3–5 rats/group). *P < 0.05, **P < 0.01 compared to vehicle controls, analyzed with unpaired t test.

and cerebral cortex (**Figure 4C**) that was reduced in rats treated with ANXA1sp at day 1 and 7 post operation.

In Vivo and *In Vitro* Modulation of NF-κB Activity by ANXA1sp

We previously demonstrated that both non-steroidal and steroidal anti-inflammatory drugs such as glucocorticoids and nitric oxide–aspirin, induce expression of ANXA1, which directly binds to the NF-κB p65 subunit, and thereby inhibits its activation in cancer models (26). Here, we found that NF-κB activity in the brain was significantly attenuated in the ANXA1sp-treated rats at 6 h after CPB/DHCA (**Figure 5A**). Notably, levels of NF-κB increased after CPB/DHCA (with vehicle) compared to sham and naïve rats (Figure S1 in Supplementary Material), hence we focused this study on the comparison between vehicle-treated and ANXA1sp-treated rats. Confocal microscopy in the cerebral cortex revealed ANXA1 co-localized with nuclear NF-κB p65 after treatment, suggesting a possible similar mechanism as earlier described in cancer models (26) (**Figure 5B**). Thus, we assessed protein levels of ANXA1 by western blot and found a significant increase in the expression following peptide administration compared to naïve- and vehicle-treated rats (**Figure 5C**, $P < 0.01$ vs naïve and $P < 0.05$ vs vehicle, respectively).

Given the effects of ANXA1sp on microglial activation after CPB/DHCA we then used immortalized murine microglial cell line BV2. BV2 cells were pretreated with ANXA1sp for 1 h, and then exposed to 2 h OGD (hypoxia) followed by 24 h reoxygenation, to mimic I/R injury from CPB in the rat model. No changes were observed in ANXA1sp-treated cells under normoxic or sham conditions. However, in cells subjected to hypoxia, we discovered that cell survival (by MTT assessment) was maximal after pretreatment with 30 μM ANXA1sp (Figure S2 in Supplementary Material). Thus, we used this dosage for the remainder of the *in vitro* studies reported here.

Cells pretreated with ANXA1sp had lower levels of NF-κB DNA binding activity (based on the gel electrophoresis mobility shift assay) at all time points after OGD (**Figure 6A**). Using confocal microscopy we also observed increased levels of ANXA1 and co-localization with NF-κB p65 (**Figure 6B**). These findings corroborated our *in vivo* results. Further, both necrotic (**Figure 7A**, 2.58 ± 0.40 vs 1.29 ± 0.53, $P < 0.01$) and apoptotic (**Figure 7B**, 1.74 ± 0.12 vs 1.23 ± 0.18, $P < 0.01$) cell death following hypoxia reoxygenation were reduced after pretreatment with 30 μM ANXA1sp assessing oligosome formation as an index of DNA fragmentation by ELISA (30). TNFα release in culture media was also suppressed after pretreatment with ANXA1sp (**Figure 7C**, 3.21 ± 0.30 vs 1.51 ± 0.15, $P < 0.001$).

Neurological and Neurocognitive Outcomes after CPB/DHCA and ANXA1sp Treatment

Finally, we evaluated neurobehavioral changes after CPB/DHCA and ANXA1sp treatment. Neurologic scores were assessed on day 3 and day 7 post operation and showed improved sensory-motor functions (including processing involving retrosplenial and posterior parietal cortex) in ANXA1sp-treated rats compared to vehicle-treated rats, with scores returning to baseline by day 7 post operation (**Figure 8A**). We used the Morris water maze to evaluate spatial learning and memory (involving hippocampal function). Fisher's least significance difference *post hoc* test showed the cognitive function was significantly improved at day 3 of the water maze (**Figure 8B**, $P < 0.003$).

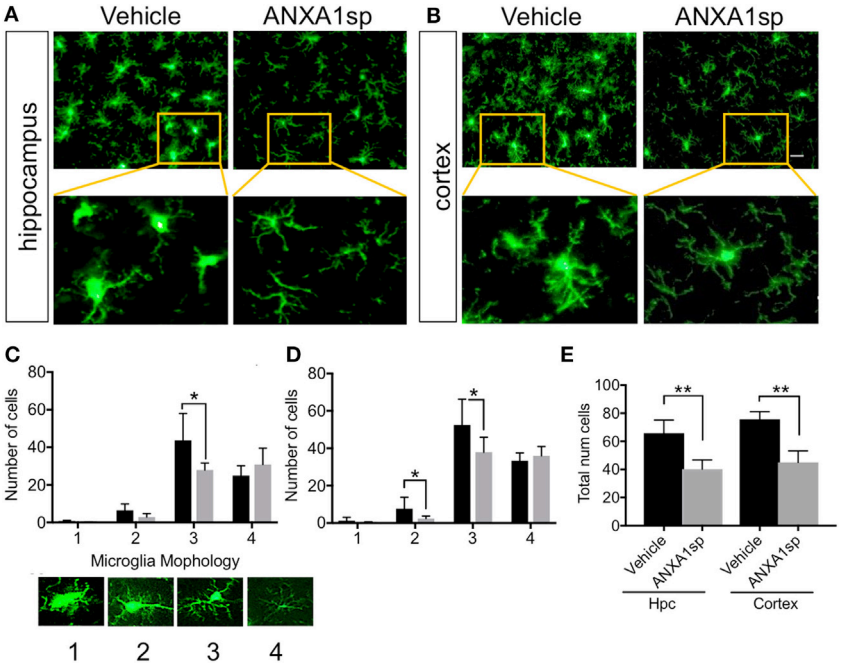

FIGURE 3 | Microglial activation after cardiopulmonary bypass/deep hypothermic circulatory arrest and ANXA1sp or vehicle treatment. ANXA1sp significantly improved microglial morphology both in the hippocampus **(A)** and cerebral cortex **(B)** 24 h after CPB/DHCA. **(C,D)** Microglial morphology was quantified based on four morphological subtypes: 1. round/amoeboid microglia; 2. stout microglia; 3. microglia with thick long processes; and 4. microglia with thin ramified processes. **(E)** Overall microglial numbers in the hippocampus and cerebral cortex were reduced after surgery in ANXA1sp-treated rats. Scale bar: 20 μm. Data are presented as mean ± SD (n = 3–5 slides/tissue section from five rats per group). *P < 0.05 compared to vehicle controls, analyzed with two-way ANOVA Sidak's multiple comparisons test **(C,D)** and **P < 0.01 with unpaired t test **(E)**.

DISCUSSION

In this study, we evaluated the potential of a bioactive ANXA1 peptidomimetic to confer neuroprotection after cardiac surgery. Our findings show that systemic administration of ANXA1sp reduced brain and circulating levels of pro-inflammatory markers while improving neurocognitive outcomes following CPB/DHCA in rats. In particular, treated animals displayed significant decreases in microglial activation, NF-κB activation, and release of pro-inflammatory mediators in the CNS and systemically, as well as modulation of cell death in different brain regions after CPB/DHCA.

Annexin A1 was the first described member of the annexin superfamily, which includes 13 mammalian proteins with distinct biologic roles (35). It is widely expressed in different organs, and signals *via* the lipoxin A4 receptor (ALX). ANXA1 has profound effects on innate immunity including regulation of glucocorticoid activity by inhibiting eicosanoid synthesis and phospholipase A2 (PLA2) (36). The N-terminal domain of this molecule is pivotal in mediating several of its biologic functions as well as signaling *via* FPR receptors (37). These actions result in potent immunoregulatory effects, especially on inflammatory phagocytes and neutrophils by inhibiting their accumulation and migration. After splanchnic artery occlusion and reperfusion injury, treatment with ANXA1 N-terminal peptidomimetic (Ac_{2-26}) reduced MPO activity and neutrophil infiltration into the reperfused tissue and thus, improved outcome after shock (38). Recently, using a model of middle cerebral artery occlusion and reperfusion in mice that recapitulates warm focal I/R injury, Vital et al. showed attenuation of cerebrovascular injury after administration of ANXA1 Ac_{2-26} (39). This study highlighted the importance of FPR2/ALX on neutrophils as a central player in controlling formation of neutrophil-platelet aggregates in the cerebral microcirculation post-I/R as a central mechanism for resolving neuroinflammation. Our findings complement those of Vidal et al. in a rat model of cardiac surgery-associated cold global cerebral I/R, with postoperative MPO levels significantly reduced in brain and plasma following ANXA1sp treatment (**Figures 1B** and **2B**), suggesting a similar mode of action and attenuation of neutrophil infiltration into the CNS.

Changes in endothelial function have been described after cardiac and non-cardiac surgery (40–42), with translational relevance as well as significant implications related to the pathogenesis of postoperative delirium and cognitive dysfunction. Indeed, after CPB/DHCA, we previously reported changes in blood–brain barrier (BBB) permeability and tight junction protein expression in purified CNS capillaries (43). Although we cannot ascertain whether ANXA1sp exerts systemic and/or central effects, BBB opening after CPB provides direct access to the brain and several putative cellular targets. ANXA1sp is expressed by different cell types in the CNS, including neurons, microglia, and astrocytes (44). Moreover, ANXA is a critical regulator of BBB integrity by stabilizing tight junction expression and is often downregulated in disorders such as multiple sclerosis (45) and Alzheimer's disease (46).

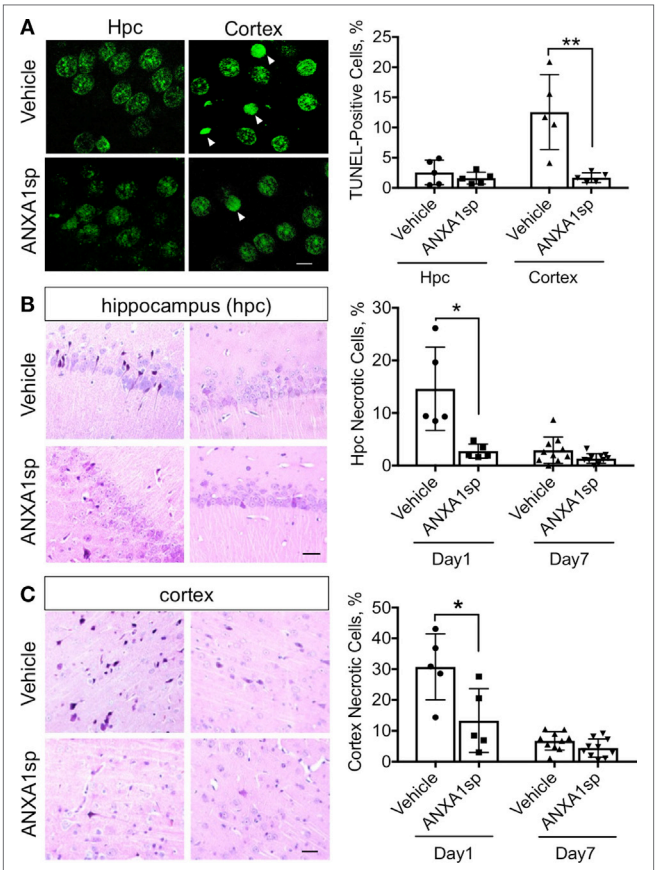

FIGURE 4 | Apoptosis and necrosis in the CNS after cardiopulmonary bypass/deep hypothermic circulatory arrest and ANXA1sp or vehicle treatment. **(A)** Terminal deoxynucleotidyl nick-end labeling staining and quantification of apoptotic bodies. Apoptosis was significantly reduced in the cerebral cortex of ANXA1sp-treated rats on day 1 post operation. **(B,C)** Necrosis was detected by acid fuchsin–celestine. Necrosis was reduced in a time-dependent fashion in the hippocampus and cerebral cortex of ANXA1sp rats, with levels returning to baseline by day 7 post operation. Arrowheads in panel A identify pyknotic positive cells. Scale bar: 20 μm. Data are presented as mean ± SD (n = 5–10 slides/tissue section from five rats per group). *P < 0.05, **P < 0.01 compared to vehicle controls, analyzed with unpaired t test.

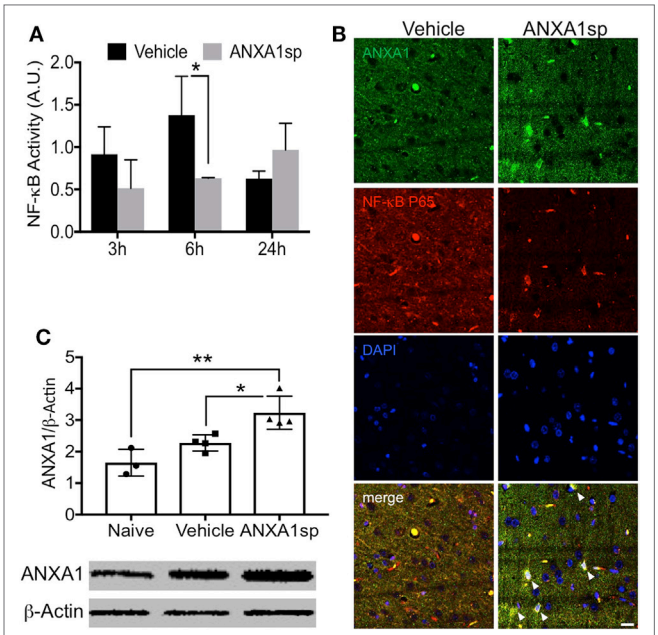

FIGURE 5 | Modulation of cerebral NF-κB DNA binding activity and expression of ANXA1 after cardiopulmonary bypass/deep hypothermic circulatory arrest. **(A)** NF-κB DNA binding activity was significantly reduced in brain homogenates from ANXA1sp-treated rats at 6 h post reperfusion. **(B)** Co-localization of ANXA1 and NF-κB p65 (arrowheads) was visualized by double immunofluorescence staining and confocal microscopy. **(C)** ANXA1sp promoted expression of cerebral ANXA1 in rats at 24 h following CPB/DHCA. Scale bar: 20 μm. Data are presented as mean ± SD (n = 3–5 rats/group). *P < 0.05, **P < 0.01 compared to vehicle controls, analyzed with unpaired t test **(A)** or one-way ANOVA with Tukey's multiple comparisons test **(C)**.

Here, we found sound evidence for potent neuroprotective effects of ANXA1sp, including reduction in neuroinflammation (**Figure 1**), microglial activation (**Figure 3**), cell death (**Figure 4**), and overall improved neurobehavioral outcomes (**Figure 8**). Some of these effects may be systemically mediated with direct actions at the inflammatory site given the peripheral route of drug administration in this study. In fact, plasma levels of pro-inflammatory cytokines including TNF-α and IL-6 were reduced as early as 3 h after reperfusion, with levels returning to baseline by 24 h (**Figure 2**). This is consistent with the anti-inflammatory effects, as well as direct myocardial, protection of ANXA1 in other models of cardiac injury (47, 48).

Our current study focused on remote effects of CPB and DHCA on the CNS by evaluating a potential role for ANXA1sp in resolving neuroinflammation. We found that cell necrosis and apoptosis were reduced in different brain regions after CPB/DHCA in ANXA1sp-treated rats. In an earlier study on ANXA1, similar findings were reported after spinal cord injury through inhibition of caspase-3 and PLA2 activity (49). Further, ANXA1 in microglia facilitates clearance of apoptotic neurons after contact with a neurotoxin (50).

Microglia are central to the onset and progression of inflammation in the CNS. Although the function and exact role of these cells is highly dependent on activation state, reactive microglia contribute to neuroinflammation and a maladaptive response that contributes to neuronal dysfunction (33). After exposure to CPB and DHCA, we found changes in numbers and morphology of microglia in different brain areas, including the hippocampus (**Figure 3**). Microglial activation has been reported in orthopedic (51), vascular (52), abdominal (53), and cardiac (54) surgery-induced neurocognitive disorders. Although the mechanisms that contribute to microglial activation are multifactorial and include both humoral and neuronal signaling, modulation of ANXA1 is a promising target for intervention.

Annexin A1 is abundant in microglia (55), and we found that ANXA1sp boosts expression of ANXA1 in these cells possibly facilitating resolution of neuroinflammation (**Figures 3** and **7**). Our *in vivo* and *in vitro* data demonstrate a key role for NF-κB activation in microglial cells, and NF-κB activation represents a key regulatory gene for *de novo* synthesis of pro-inflammatory

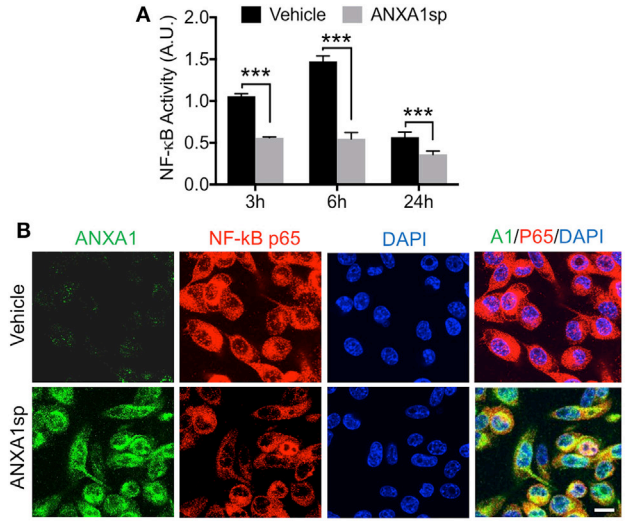

FIGURE 6 | Modulation of NF-κB activation after oxygen–glucose deprivation in BV2 cells pretreated with 30 μM ANXA1sp or vehicle. **(A)** NF-κB activity based on the gel electrophoresis mobility shift assay was significantly reduced in a time-dependent fashion in ANXA1sp-treated cells. **(B)** After 24 h reoxygenation, co-localization of annexin A1 (ANXA1) and NF-κB p65 subunits was visualized by double immunofluorescence staining and confocal microscopy. Scale bar: 20 μm. Data are presented as mean ± SD ($n = 3$ independent experiments). ***$P < 0.001$ compared to vehicle controls, analyzed with unpaired t test.

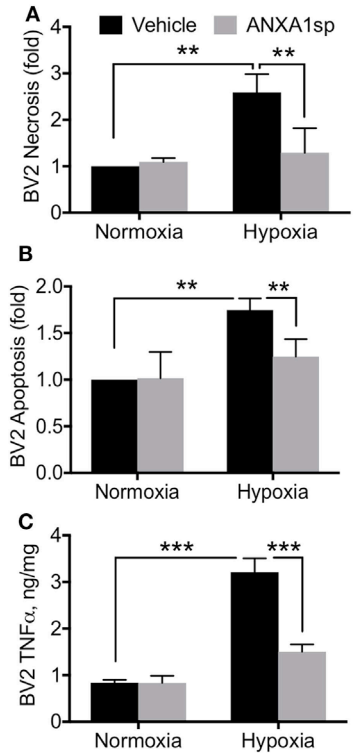

FIGURE 7 | ANXA1sp reduced microglial cell death and TNFα. BV2 cells were pretreated with ANXA1sp or vehicle for 1 h, and then subjected to 2 h oxygen–glucose deprivation (OGD) followed by reoxygenation. Cell necrosis **(A)** and apoptosis **(B)** were assessed by ELISA assay. Following OGD, 30 μM ANXA1sp prevented both cell necrosis and apoptosis in ANXA1sp-treated BV2 cells. **(C)** TNFα levels in cell culture medium were also restored in cells pretreated with ANXA1sp. Data are presented as mean ± SD ($n = 3$ independent experiments). 30 μM ANXA1sp was selected as the optimal treatment based on the dose response (Figure S2 in Supplementary Material). **$P < 0.01$, ***$P < 0.001$ compared to vehicle controls, analyzed with unpaired t test or one-way ANOVA with Tukey's multiple comparisons test.

cytokines as well as cell death processes. Although, we cannot ascertain if microglia are the primary and sole target of ANXA1sp, this small peptidomimetic is likely to exert several effects both on peripheral and central inflammatory processes. Importantly, post-I/R NF-κB activation was dampened after treatment with ANXA1sp (**Figures 5**–**7**), and TNF-α and IL-6 levels were reduced accordingly in both brain tissue and plasma in a time-dependent fashion after CPB/DHCA (**Figures 1** and **2**). These findings suggest then, that modulation of NF-κB activation may reduce neuronal damage and improve behavioral outcomes after cardiac surgery.

Several protective effects of ANXA peptidomimetics have been described in different models [reviewed in Ref. (56)]. We previously reported anti-inflammatory effects of our ANXA1sp tripeptide on NF-κB inhibition in models of colon cancer (26). In this surgical model, ANXA1sp not only reduces NF-κB DNA binding activity, but also increases levels of ANXA1, which can bind directly to NF-κB p65 to further inhibit its transcriptional activity (**Figure 5**). These findings are relevant and possibly unique since the N-terminal domain sequence of this tripeptide (Ac-Gln^{10}–Ala^{11}–Trp^{12}) has been shown to have greater binding affinity for FPR2/ALX (57). In general, FPR2/ALX shows high promiscuity in terms of ligand recognition, and thus possesses very complex functional properties including both promotion of resolution and pro-inflammatory effects (58). This may be important because even though other peptidomimetics, including nanoparticles encapsulating ANXA1 mimetic peptide Ac_{2-26} (59), SuperAnxA1 (60), and CR-$AnxA1_{2-28}$ (14), regulate efferocytosis and neutrophil activity, ALX agonists can activate other

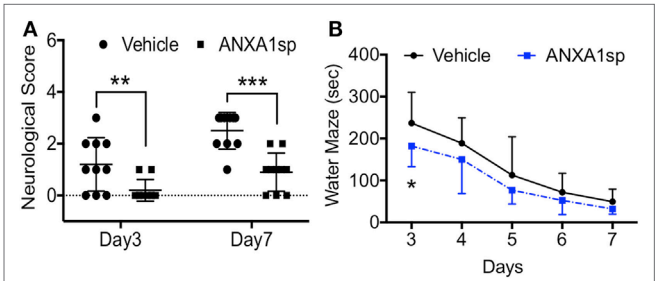

FIGURE 8 | Neurocognitive outcomes after cardiopulmonary bypass/deep hypothermic circulatory arrest and ANXA1sp or vehicle treatment. **(A)** Neurologic scores were assessed on day 3 and day 7 post operation. Sensory-motor functions were significantly improved at both time points in ANXA1sp-treated rats. **(B)** Morris water maze testing was performed daily from day 3 through day 7 post operation. Learning abilities were significantly improved in ANXA1sp rats on day 3 post operation. Data are presented as mean ± SD ($n = 10$ rats/group). *$P < 0.003$, **$P < 0.01$, ***$P < 0.001$ compared to vehicle controls. Mann–Whitney U test was applied for the neurological score comparisons at each recovery interval. Repeated measures ANOVA with Fisher's least significance difference *post hoc* test was used for Morris water maze analysis.

specialized pro-resolving signaling and extend therapeutic effects (61). Currently, surgery-induced neurologic complications such as delirium and postoperative cognitive dysfunction have no effective therapy. Between 14% (62) and 50% (63) of cardiac surgery patients suffer from postoperative neurocognitive impairments, with effects lasting up to several years (64). Therapeutic strategies that reduce and promote resolution of neuroinflammation may limit these complications without significantly affecting homeostatic and reparative processes (65).

In the current study, ANXA1sp was systemically administered, and thus, we cannot verify the exact site of action of this small peptide. Although we observed significant anti-inflammatory changes both systemically and in the CNS, we cannot yet assign causal effects. For our *in vitro* experiments we used an immortalized murine microglial cell line (BV2) as a suitable alternative to primary microglia (50, 66), however, the role ovvf FPR2/ALX signaling in microglial activation or monocytes trafficking through the BBB were not formally evaluated in this study. Given the critical role of ANXA1 in regulating BBB integrity, it is possible that regulation of endothelial function after surgery protects against secondary effects in the CNS. We used a clinically relevant rat model of CPB and DHCA to study secondary effects on the CNS, yet to allow for long-term survival, median sternotomy, direct cardiac cannulation, and opening of cardiac cavities were not performed. Extended exposures to anesthesia, such as sevoflurane, without surgical manipulation downregulated ANXA1 expression in endothelial cells, which contributed to cognitive deficits (67). Here, we found that expression of ANXA1 was also increased following ANXA1sp treatment (**Figure 5C**), and although the signaling mechanisms *via* FPR2/ALX must be further refined. It remains unclear if treatment with this ANXA1 peptidomimetic boosts endogenous levels of ANXA1 or simply contributes to the existing pool of pro-resolving factors, thus facilitating the resolution cascade.

Taken together these findings demonstrate the potential for exploiting innate neuroprotective mechanisms to minimize cerebral I/R damage in general and that novel stable activators of this pathway may serve as resolution-targeting strategies to prevent or limit perioperative cerebral injury and associated neurocognitive complications.

ETHICS STATEMENT

The experimental protocol was approved by the Duke University Animal Care and Use Committee. All procedures were in accordance with the guidelines of the National Institutes of Health for animal care (Guide for the Care and Use of Laboratory Animals, Health and Human Services, National Institutes of Health Publication No. 86-23, revised 1996).

AUTHOR CONTRIBUTIONS

ZZ, QM, and MP designed and performed research; BS, DL, GM, JM, MP, and NT contributed new reagents/analytic tools; ZZ, QM, MP, and NT analyzed data; ZZ, QM, and NT wrote the manuscript; ZZ and QM contributed equally to this study. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01050/full#supplementary-material.

FIGURE S1 | Expression of NF-κB in naive, sham, and cardiopulmonary bypass with deep hypothermic circulatory arrest.

FIGURE S2 | Cell viability measured by MTT assay at 24 h OGD/reoxygenation.

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REVIEW
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Impaired Resolution of Inflammation in Alzheimer's Disease: A Review

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Alzheimer's disease (AD) remains the leading cause of dementia worldwide, and over the last several decades, the role of inflammation in the pathogenesis of this neurodegenerative disorder has been increasingly elucidated. The initiation of the acute inflammatory response is counterbalanced by an active process termed resolution. This process is designed to restore homeostasis and promote tissue healing by the activation of neutrophilic apoptosis, promotion of neutrophil clearance by macrophages, and increasing anti-inflammatory cytokine levels, while concurrently leading to a diminution in pro-inflammatory mediators. The switch from the initiation to the resolution phase of inflammation is initially characterized by increased production of arachidonic acid-derived pro-resolving lipoxins and decreases in pro-inflammatory prostaglandin and leukotriene levels, subsequently followed by increases in specialized pro-resolving lipid mediators derived from omega-3 fatty acids (ω-3 FAs). There is mounting evidence that in AD, the resolution of inflammation is impaired, resulting in chronic inflammation and the exacerbation of the AD-related pathology. In this review, we examine preclinical and clinical evidence supporting the hypothesis that AD is a neurodegenerative disorder where the impairment or failure of resolution contributes to the disease process. Moreover, we review the literature supporting the potential therapeutic role of ω-3 FAs and specialized pro-resolving lipid mediators in the management of the disease. Lastly, we highlight areas that could strengthen the association of failed resolution to AD and should, therefore, be the focus of future scientific investigations in this research field.

Keywords: Alzheimer's disease, resolution, inflammation, beta-amyloid, tau, resolvins, specialized pro-resolving lipid mediators, omega-3 fatty acids

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INTRODUCTION

With an estimated global prevalence of 46.8 million affected individuals in 2015, Alzheimer's disease (AD) remains the leading cause of dementia worldwide (1). The majority of new AD cases are sporadic and late-onset in nature, and without a major therapeutic breakthrough, it is estimated that the prevalence will quadruple by the middle of this century (2), ultimately reaching a global prevalence of approximately 131.5 million (1). The major neuropathological hallmarks of AD include extracellular senile plaques composed of aggregates of beta-amyloid (Aβ) protein (3), and intraneuronal neurofibrillary tangles made up of aggregated tau protein (4, 5). Although the German psychiatrist and neuropathologist Alois Alzheimer described the neuropathological hallmarks of AD over a

century ago (6), the role of inflammation in the pathogenesis of the disease was not fully appreciated until several decades later.

Recent work has demonstrated that the termination of the acute inflammatory response is dependent on an active process termed "resolution" (7–9). In this review, we will review the scientific evidence linking the impairment or failure of resolution to AD, the specific neuropathological consequences of resolution failure in this neurodegenerative disorder, and the potential for the restoration of resolution to serve as a therapeutic target in AD.

NEUROINFLAMMATION AND AD

Neuroinflammation has been implicated in playing a key role in the pathogenesis of AD, with studies suggesting various mechanisms including astrocyte (10–13) and microglial activation (14–18), increases in pro-inflammatory molecules such as cytokines and chemokines [reviewed in Ref. (19)]. Moreover, there is evidence to suggest that the activation of endothelial cells of the neurovascular unit, oligodendrocytes, and even neurons may be involved [reviewed in Ref. (20)]. In AD patients, the role of neuroinflammation is clinically supported by PET studies demonstrating microglial activation (21–23), increased serum (24–26), and brain (27, 28) pro-inflammatory cytokine levels as well as the downregulation of anti-inflammatory molecules in postmortem brain tissue (29). Moreover, although the CNS was once considered an immunologically privileged site, it is now recognized that peripheral inflammation can exacerbate the inflammatory environment of the brain and contribute to chronic neuroinflammation and neurodegeneration (30, 31).

RESOLUTION OF INFLAMMATION: A VITAL PROCESS FOR THE RESTORATION OF TISSUE HOMEOSTASIS

The initiation of the acute inflammatory phase, usually in response to trauma, infection, tissue injury, neoplasia, or other major homeostatic stressors, has been well characterized by the increased release of pro-inflammatory mediators such as prostaglandins and leukotrienes, leading to polymorphonuclear (PMN) leukocyte recruitment and monocyte-macrophage proliferation. It was initially believed that the acute response to inflammation passively dissipated over time; however, it has been more recently appreciated that acute inflammation is actually kept in homeostatic balance by resolution (32, 33), which ultimately results in the clearance of recruited granulocytes and a restoration of pre-activation immune profiles [reviewed in Ref. (7, 9)]. Failure of resolution has been associated with the development of chronic inflammation (34), which has been implicated in the pathogenesis of many diseases including asthma, periodontitis, rheumatoid arthritis, ulcerative colitis, multiple sclerosis, and AD [reviewed in Ref. (9)].

The resolution of inflammation is initiated by a change in eicosanoid signaling that shifts from a pro-inflammatory to a pro-resolution, anti-inflammatory state, which is characterized by the biosynthesis of specific mediators. Sophisticated advances in lipidomics and metabolomics, spearheaded by seminal investigations by Serhan and his colleagues, have resulted in the identification and functional classification of these specialized pro-resolving mediators (SPMs) that drive resolution (7–9, 35–38). During the initiation phase of the acute inflammatory response, mediators derived from arachidonic acid become up-regulated and contribute to changes in vascular permeability and PMN leukocyte recruitment (39). However, the generation of these pro-inflammatory autacoids is eventually terminated by subsequent dynamic changes in prostaglandins E2 and D2 (39). This can be seen as a *switch* where increased levels of SPMs, including pro-resolving lipoxins [e.g., Lipoxin A4 (LXA4)], diminish inflammatory molecules such as prostaglandins, leukotrienes, and cytokines (**Figure 1A**). Pivotal to this *switch* is a class of lipid mediators, or SPMs, which are biosynthesized from the omega-3 fatty acids (ω-3 FAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and regulate acute inflammation by promoting active resolution (**Figure 1B**). These SPMs include the resolvin D series compounds, protectins/neuroprotectins, and maresins, which are derived from DHA, as well as the resolvin E series compounds derived from EPA (**Figure 1B**) [reviewed in Ref. (34, 38, 40)]. The pro-resolving effects of the lipoxins and the ω-3 FA-derived SPMs collectively exert key features of resolution: suppression of PMNs, decrease vascular permeability, promotion of non-phlogistic monocyte recruitment, and increase macrophage-mediated clearance of

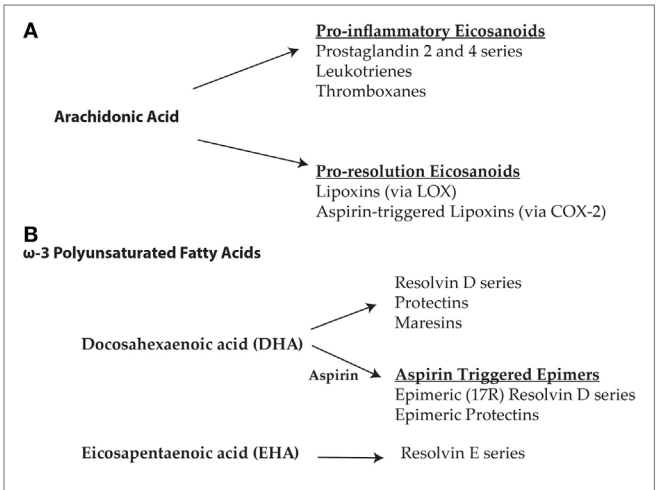

FIGURE 1 | **(A)** The initiation phase of acute inflammation is characterized by an increase in the arachidonic acid-derived pro-inflammatory eicosanoids including prostaglandins, leukotrienes, and thromboxanes. Increases in prostaglandins eventually lead to a change in the biosynthesis of eicosanoids derived from arachidonic acid resulting in the formation of pro-resolution lipoxins via lipoxygenase-associated pathways. In the presence of aspirin, cyclooxygenase-2 is acetylated leading to the formation of the 15-epi-lipoxins (aspirin-triggered lipoxins). The aspirin-triggered isoforms are more resistant to degradation and hence more bioactive than native specialized pro-resolving mediators (SPMs). **(B)** Resolution is further propagated by the synthesis of SPMs derived from the omega-3 polyunsaturated fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA). SPMs derived from DHA include the resolvin D series, protectins, and maresins, as well as the aspirin-triggered epimeric (17R) forms of the resolvin D series and protectins. EPA derived SPMs include the resolvin E series.

apoptotic PMNs. However, during chronic inflammatory states, including neurodegeneration, this balance is disrupted and the predominance of arachidonic acid-derived pro-inflammatory lipid mediators contributes to the impaired resolution of inflammation and the persistence of a generalized pro-inflammatory state.

PRECLINICAL STUDIES SUPPORTING IMPAIRED RESOLUTION IN AD

In Vivo Studies

Aging is associated with increased inflammation (41–43), and this, in itself, is of critical importance as it also is the major risk factor for the development of late-onset AD. Indeed, it has been recently demonstrated in BalbC mice that aging is associated with a delay in the resolution of inflammation. This is characterized by a greater increase and slower clearance of recruited PMNs following an acute inflammatory challenge, resulting in higher levels of pro-inflammatory cytokines and defective SPM production (44). Thus, given the impact that aging has on the development of AD, it is reasonable to hypothesize that age-related defects in resolution and "inflammaging" (45) may potentially contribute to the development and propagation of this neurodegenerative condition.

Over the last several years, there has been an increase in preclinical studies demonstrating that failed resolution plays a role in the development of AD pathology. Wang et al. demonstrated in senescence-accelerated mice-prone 8 (SAMP8), a murine model of accelerated aging that spontaneously exhibits β-amyloid (Aβ) overproduction, tau hyperphosphorylation, oxidative stress damage, and cognitive decline (46) that aging was indeed associated with a pro-inflammatory state (47). However, when compared with similarly aged 9-month-old senescent-accelerated mouse resistant 1 (SAMR1) mice, a mouse strain that displays normal aging without cognitive decline, SAMP8 mice did not exhibit any difference in LXA4 or resolvin D1 (RvD1) levels, despite a greater degree of inflammation in SAMP8 mice. Levels of ALX/FPR2 (*N*-formyl peptide receptor 2), the G protein-coupled receptor for Annexin-A1, LXA4, and RvD1, were also increased in SAMP8 mice but remained at levels similar to that of SAMR1 mice. This suggests that, despite the increased inflammation observed in SAMP8 mice, there is not a commensurate increase in SPMs. Hence, changes in ALX/FPR2 receptor density may actually be a compensatory response. Interestingly, in 9-month-old SAMP8 mice, these same investigators observed lower hippocampal levels of leukocyte-type 12-lipoxygenase (L12-LOX), the initial enzyme involved in the production of LXA4 and RvD1 from AA and DHA, respectively, when compared to similarly aged SAMR1 mice. Furthermore, L12-LOX co-localized with Aβ in the hippocampus. Interestingly, the lower L12-LOX levels positively correlated with increased levels of phosphorylated tau at the Ser^{202}/Thr^{205} (AT8) phosphoepitope, suggesting that the impaired resolution response may impact the degree of Aβ and tau pathology that manifests with age in this strain.

Recent studies, utilizing lipoxin treatment in transgenic mice that develop AD-like pathology, have also provided compelling preclinical evidence supporting the role of impaired resolution in the development of AD pathology (48, 49). Lipoxins, particularly LXA4 and their aspirin-triggered (AT) carbon-15 (15R) epimers, are potent promoters of resolution by antagonizing pro-inflammatory mediators *via* activation of ALX/FPR2 receptors, resulting in decreases in leukocyte recruitment, NF-κB activation, superoxide generation, and longer-lasting effects on pro-inflammatory chemokine/cytokine production (35, 50–53). Aspirin has been shown to modulate lipoxin biosynthesis by yielding 15R epimerization products termed AT lipoxins, thus making it more resistant to inactivation and further promoting resolution signaling (52). In Tg2576 mice, which harbor the Swedish double mutation in human amyloid precursor protein leading to the rapid development of Aβ-related pathology, the administration of AT-LXA4 has been shown to reduce the activation of NF-κB and levels of pro-inflammatory cytokines as well as increase levels of the anti-inflammatory cytokine IL-10, ultimately resulting in an alternative microglial phenotype (48). This subsequent change in microglial phenotype was associated with improved phagocytosis, increased Aβ clearance, decreased synaptotoxicity, as well as cognitive improvement in the Tg2576 mice. More recently, Dunn et al. demonstrated in triple transgenic AD (3xTg-AD) mice, a transgenic mouse strain that expresses the Aβ-processing related mutations APP_{SWE} and $PS1_{M146V}$ as well as mutant, pro-aggregant tau (tau_{P301L}), thus respectively developing accelerated senile plaque and neurofibrillary tangle pathology, that brain LXA4 levels significantly decreased with age. This decrease in LXA4 levels was more pronounced in the 3xTg-AD mice than in non-transgenic mice; moreover, an 8-week treatment with AT-LXA4 reduced cognitive impairment, Aβ levels, and tau phosphorylation (49). Interestingly, the reduction in brain tau phosphorylation was mediated by decreased activation of the tau-related kinases, GSK-3β and p38 MAPK. Taken together, these preclinical studies support the concept that increased lipoxin signaling restores resolution physiology that, in turn, may serve as therapeutic means for attenuating Aβ and tau pathology as well as cognitive decline.

There is now growing interest as to whether the resolvins can actually attenuate AD pathology and modulate cognitive deficits, especially as the potentially neuroprotective effects of SPMs are increasingly elucidated. Using a mouse model of postoperative cognitive dysfunction following orthopedic surgery, acute administration of AT RvD1 prevented neuronal dysfunction and cognitive impairment by regulating long-term potentiation, and astrocyte activation (54). Using this same murine neuroinflammation model, we are currently conducting studies to determine whether the resolvins or other SPMs can attenuate changes in tau pathology following surgery-induced neuroinflammation. Recently, DHA-derived neuroprotectin D1 (NPD-1) has been observed to exert potent neuroprotective effects in multiple models of CNS injury and neurodegeneration by modulating synaptic plasticity, dendritic spine morphology, and microglia activation [reviewed in Ref. (55)]. Other SPMs, including resolvin E1 (RvE1), have also been implicated in the regulation of excitotoxic signaling, synaptic transmission, and neuroinflammation in models of inflammatory and postoperative pain (56). Although the exact mechanisms of resolvins on neuron–glia interactions

have been only partly elucidated, SPMs may provide novel strategies to modulate and possibly prevent the onset and progression of neurodegenerative conditions including AD.

In Vitro Studies

Human CHME3 microglia incubated with $A\beta_{42}$, when compared with lipopolysaccharide (LPS) stimulation, have been recently observed to manifest decreased phosphorylation at serine 523 of 5-lipoxygenase (57), a key enzyme involved in the regulation of leukotriene and lipoxin production. This decreased phosphorylation at serine 523 resulted in increased leukotriene production and decreased lipoxin formation, which are changes consistent with disrupted resolution. Interestingly, $A\beta_{42}$ or LPS did not alter the levels of LXA4 or RvD1 in the cell culture medium; furthermore, in contrast to the increase observed with LPS, $A\beta_{42}$ had no effect on receptor levels of ALX/FPR2, the receptor for LXA4 and RvD1. Therefore, when compared to the pro-inflammatory stimulus LPS, it appears that $A\beta_{42}$ is associated with changes that are consistent with impaired resolution and the potential propagation of a chronic inflammatory state.

Zhu et al. more recently demonstrated that LXA4, maresin 1, protectin DX, and RvD1 decreased staurosporine-induced apoptosis in human SH-SY5Y neuroblastoma cells (58). Furthermore, maresin 1 was observed to be particularly protective in terms of Aβ-related pathology, as this SPM increased microglial phagocytosis of $A\beta_{42}$ as well as attenuated $A\beta_{42}$-mediated activation of human CHME3 microglia. Of note, incubation of human CHME3 microglia with maresin 1 also attenuated levels of the pro-inflammatory biomarkers CD11b (activated), MHC II, and CD86. Hence, at least *in vitro*, it appears that maresin 1 may have a greater potential for attenuating certain aspects of Aβ-associated pathology in AD when compared to other SPMs.

Hitherto, most of the *in vivo* and *in vitro* investigations examining the impact of AD-associated neuropathology on resolution pathways have primarily focused on Aβ pathology. Indeed, although AD is a secondary tauopathy, the role of impaired resolution on the progression of tau pathology and *vice versa* has been the focus of fewer studies compared to Aβ-related pathology. Whether failed resolution leads to an exacerbation of neurofibrillary pathology and the subsequent impairment of resolution, either directly or via the potentiation of Aβ-mediated mechanisms that suppress resolution, definitely warrants further investigation.

CLINICAL EVIDENCE SUPPORTING IMPAIRED RESOLUTION PHYSIOLOGY IN HUMANS WITH AD

Although age is the greatest risk factor for developing late-onset AD, only recently have investigators started to appreciate the impact of aging on resolution in humans. Gangemi et al., measured urine LXA4 and pro-inflammatory leukotriene levels in 30 healthy humans who were divided into three average age groups: 43.5, 77.9, and 102.5 years (59). Compared to the youngest group, both older age groups had significant lower urinary excretion of LXA4; moreover, there was a significant decrease in the LXA4/leukotriene ratio in these older age groups. These findings suggest that, in response to a pro-inflammatory stimulus, the capacity for arachidonic acid-derived pharmacology to switch from the production of pro-inflammatory leukotrienes toward increased lipoxin formation decreases with age.

In terms of AD specifically, by examining levels of SPMs and receptor expression in human CSF and brain tissue, Wang et al. performed one of the initial human investigations demonstrating that the neurodegenerative disorder is associated with impaired resolution (60). Levels of LXA4 and RvD1 were measured in CSF collected from humans with AD, mild cognitive impairment (MCI), or subjective cognitive impairment (SCI). Interestingly, levels of LXA4 in CSF and hippocampal tissue were significantly lower in the AD group versus the MCI and SCI groups; whereas, no group-related differences in hippocampal or CSF RvD1 levels were observed. Of note, there was a positive correlation between CSF LXA4 and RvD1 levels and cognitive performance as measured by the mini-mental state examination (60). In this same investigation, when compared with control subjects, immunohistochemical analyses of hippocampal tissue from AD patients revealed higher levels of the SPM receptors, ALX/FPR2 and ChemR23, the latter a receptor for RvE1. They also observed increased levels of 15-LOX-2, a key enzyme involved in the production of LXA4, as well as decreased levels of IL-10, an anti-inflammatory cytokine associated with the resolution of inflammation (61, 62).

A more recent study by Zhu et al. has provided additional evidence that resolution is impaired in humans with AD (58). These investigators measured SPM levels in the entorhinal cortex of AD patients and age-matched controls at 18–21 h *post mortem*. Compared to age-matched controls, levels of maresin 1, protectin 1, and resolvin D5 were decreased, while levels of prostaglandin D_2 were decreased in the entorhinal cortex of patients with AD. Again, these changes are consistent with an impairment of resolution in AD and the predominance of a chronic inflammatory state.

ω-3 FAs AND AD

As SPMs are biosynthesized from ω-3 FAs (8, 9, 34, 37, 38, 40, 63), the question of whether polyunsaturated fatty acids (PUFAs) impact the resolution of inflammation in AD has logically arisen. ω-3 FA-derived EPA and DHA have been demonstrated to modulate arachidonic acid metabolism in such a manner as to reduce the production of pro-inflammatory mediators. For example, DHA has been shown in astroglial cell cultures to decrease levels of pro-inflammatory thromboxane B2, 6-keto-Prostaglandin F1 alpha, and 12-hydroxyeicosatetraenoic acid (64), whereas EPA has been demonstrated to decrease the pro-inflammatory arachidonic acid derivatives, prostaglandin E2 and leukotriene B4 (65, 66). Patients with AD have lower plasma (67) and brain levels of DHA (68), suggesting that PUFA supplementation may provide beneficial effects on the neuropathology and cognitive function. Furthermore, in AD patients with moderate plaque and tangle pathology, Lukiw et al. demonstrated that levels of NPD-1, a DHA-derived protectin compound with pronounced neuroprotective and anti-inflammatory effects, were

significantly decreased in the CA1 region of the hippocampus (68). Aging itself also appears to negatively affect FA metabolism (69, 70); hence, a better understanding of the impact of ω-3 FAs on mechanisms associated with the resolution of inflammation is important to fully elucidate the potential therapeutic role of these compounds in AD.

In vitro studies have demonstrated that DHA and EPA enhance $A\beta_{42}$ phagocytosis in CHME3 human microglial cell culture (71). Moreover, this occurred in a biphasic pattern characterized by an increase at 2 h followed by a quiescent period and then another phase of activated phagocytosis at 24 h. The late phase activation of $A\beta_{42}$ phagocytosis was postulated to involve the time-dependent accumulation of SPMs such as resolvins and maresins. Levels of SPMs were not measured in cell culture to confirm this; nevertheless, the time frame is consistent with possible activation of non-phlogistic phagocytosis.

In animal studies incorporating the use of transgenic AD rodent models, ω-3 FAs have been shown to have beneficial effects on Aβ pathology, tau pathology, and neuroinflammation [reviewed in Ref. (72)]. In terms of AD-related pathology, ω-3 FAs have been observed to specifically induce the following potentially therapeutic effects: reductions in Aβ accumulation (73) and Aβ plaque density (74), changes in Aβ ratios favoring the less fibrillogenic forms of the peptide (75), protection against tau hyperphosphorylation (76), reduced inflammation (77), and improved cognitive performance (73, 74, 76). Furthermore, a systematic review and meta-analysis focusing on the impact of ω-3 FAs on cognition and AD pathology in AD animal models revealed that long-term supplementation, which was defined as a minimum of 10% of total life span, was associated with decreased Aβ levels, improved cognition, and decreased neuronal loss (78).

EFFECT OF ω-3 FA SUPPLEMENTATION IN HUMANS WITH OR AT RISK FOR AD ON MARKERS OF RESOLUTION

Despite this preclinical evidence, in humans with AD, clinical trials incorporating ω-3 FA supplementation have demonstrated more modest benefits. One of the largest trials to date is the OmegAD study, a randomized double-blind placebo-controlled study, which randomized 204 patients with mild-to-moderate AD to 1.7 g DHA and 0.6 g EPA or placebo treatment for 6 months followed by 6 months of open treatment in both groups (79). These investigators observed that ω-3 FA treatment did not alter the rate of cognitive decline; however, in a small subset of patients with the baseline mini-mental state examination scores of >27, ω-3 FA supplementation significantly decreased cognitive decline. In a subset of patients from the OmegAD study, who were treated with ω-3 FAs for 6 months, increased plasma levels

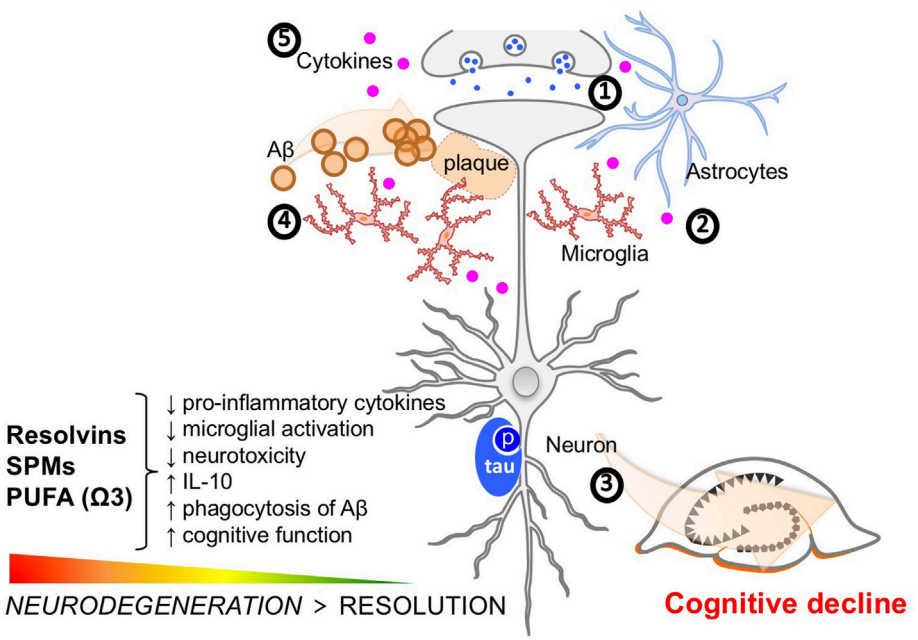

FIGURE 2 | Molecular features of Alzheimer's disease (AD) pathophysiology and key targets of resolution agonists in neurodegeneration. Clockwise from 12 o'clock (1) neurotoxicity, including dysregulated glutamate and calcium signaling, and neurotransmission imbalance contribute to synaptic dysfunction and neuronal loss; (2) glia activation, including microglia and astrocytes, interfere with immunological processes in the brain further promoting non-resolving inflammation and neurodegeneration; (3) tau phosphorylation and neurofibrillary tangle formation; (4) Aβ plaque formation are key hallmarks of the AD brain. Specialized pro-resolving mediators and strategies aimed at boosting resolution such as using omega-3 polyunsaturated fatty acid exert differential effects on these targets and provide anti-inflammatory and pro-cognitive effects in neuroinflammation/degeneration; and (5) The accumulation of Aβ may lead to the microglial accumulation and activation resulting in increases in pro-inflammatory cytokines such as interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha. These cytokine increases in the brain can subsequently lead to tau hyperphosphorylation and a pathological cycle of increased Aβ deposition and persistent microglial activation, ultimately resulting in chronic neuroinflammation and neurodegeneration.

of DHA and EPA were accompanied by a concurrent decrease in plasma arachidonic acid levels (80). In this same investigation, peripheral blood mononuclear cells from the AD patients were incubated in cell culture medium containing $A\beta_{40}$, which had been previously demonstrated to decrease production of the anti-inflammatory cytokine IL-10 (60). Interestingly, in patients treated with ω-3 FAs, levels of LXA4 and RvD1 remained stable, whereas decreases in these SPMs were observed in placebo-treated patients. Hence, in the ω-3 FA-treated group, a preservation of secreted SPM levels was evident, albeit by some unclear mechanism. In patients with MCI, ω-3 FA supplementation has been also shown to ameliorate the AD-associated impairment of Aβ phagocytosis by macrophages, increase macrophage levels of RvD1, as well as decrease cytokine transcription observed in AD patients with peripheral blood mononuclear cell evidence of pre-existing neuroinflammation [reviewed in Ref. (81)].

Nevertheless, the overall impact of ω-3 FA supplementation on the clinical course of AD in humans remains unclear. A recent systematic review of the literature of placebo-controlled clinical trials examining the impact of ω-3 FA supplementation in patients with mild-to-moderate AD concluded that there is little benefit in terms of cognitive function, daily functioning, or mental health after 6 months of treatment, whereas after 12 months of treatment, one study observed a modest improvement observed in activities of daily living (82). A more recent, larger systematic review of placebo-controlled ω-3 FA clinical trials in AD concluded that supplementation may be of benefit primarily early in the course of the disease; however, the cognitive benefits appear to be quite modest (83). Interestingly, Salem et al. reviewed two recent clinical trials examining the impact of DHA supplementation on cognitive function (84), the Memory Improvement after DHA Study (85), and the Alzheimer's Disease Cooperative Study (ADCS) (86), which examined the impact of DHA supplementation on memory and respectively demonstrated mnemonic benefit in healthy elderly subjects and no such benefit in AD patients. However, based on the observation that in the ADCS study, lower cognitive decline was observed over an 18-month period in apolipoprotein E4 (*APOE4*) allele negative patients, Salem et al. have postulated that *APOE4* may mechanistically impact the neuropathogenesis of AD by decreasing DHA transport into the brain (84). Therefore, in clinical trials focusing on the cognitive benefits of ω-3 FA supplementation in patients with or at risk for AD, *APOE4* genotype may be an important factor regulating therapeutic benefit and should be taken into consideration in the subgroup analyses.

CONCLUSION

Resolution is an active process that terminates the acute phase of inflammation and restores tissue homeostasis. Multiple preclinical and clinical investigations support the hypothesis that resolution of neuroinflammation is disrupted in AD and that this perturbation may lead to the exacerbation of AD-associated pathology and even cognitive decline (**Figure 2**). Additional studies focusing on the impact of impaired resolution on tau-related neurofibrillary pathology and function are warranted, as there is a relative dearth of tau studies versus those focusing on Aβ pathology. Furthermore, some studies have demonstrated that SPM treatment may reduce tau phosphorylation in mouse models of AD-like pathology (49).

Despite evidence that failed resolution may play a neuropathogenic role in AD, many mechanistic questions still remain unanswered. Whether the defect of resolution in AD is a consequence of a true or relative decrease in SPM levels and/or their receptor-mediated downstream effects needs to be further addressed. Other resolution-related mechanisms that may be related to AD and warrant further investigation in humans include decreased SPM production due to neuronal loss and reactive gliosis, alterations in the levels and efficiency of various enzymes responsible catalyzing SPM production, changes in arachidonic acid metabolism favoring the production of pro-inflammatory products such as leukotrienes, prostaglandins, thromboxanes, and HETE, acquired defects in SPM receptor-mediated signaling as well as decreases in adequate brain levels of the ω-3 FAs.

Although they theoretically could have beneficial effects on resolution, human studies remain inconclusive as to whether supplementation with ω-3 FAs can actually alter the clinical course of AD. Hitherto, the cognitive benefits observed with ω-3 FAs have been modest and primarily observed very early in the course of the disease. The mechanisms underlying the lack of impactful clinical benefit observed with ω-3 FAs supplementation in AD are probably multifactorial and may involve, in part, inadequate conversion of the parent ω-3 FA compounds to SPMs, alterations in SPM receptor function, and limited transport of ω-3 FA to the brain as a consequence of *APOE4* genotype. Therefore, as the SPMs themselves become more readily available for clinical investigations, human clinical trials should be considered to examine whether these compounds have a therapeutic effect in AD *via* the restoration of normal resolution physiology, and whether their direct administration obviates some of the limitations observed, thus far, with ω-3 FA supplementation.

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RW, EP, and NT conceived, wrote, and revised the manuscript.

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Neuroinflammation, Bone Marrow Stem Cells, and Chronic Pain

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Current treatments for chronic pain, such as inflammatory pain, neuropathic pain, and cancer pain are insufficient and cause severe side effects. Mounting evidence suggests that neuroinflammation in the peripheral and central nervous system (PNS and CNS) plays a pivotal role in the genesis and maintenance of chronic pain. Characteristic features of neuroinflammation in chronic pain conditions include infiltration of immune cells into the PNS [e.g., the sciatic nerve and dorsal root ganglion (DRG)], activation of glial cells such as microglia and astrocytes in the CNS (spinal cord and brain), and production and secretion of pro-inflammatory cytokines and chemokines [TNF, interleukin (IL)-1β, IL-6, CCL2, and CXCL1]. Recent studies suggest that bone marrow stem cells or bone marrow stromal cells (BMSCs) produce powerful analgesic effects in animal models of inflammatory pain, neuropathic pain, and cancer pain. We recently demonstrated that intrathecal injection of BMSCs resulted in a long-term relief of neuropathic pain for several weeks after peripheral nerve injury. Strikingly, this analgesic effect is mediated by the anti-inflammatory cytokine transforming growth factor beta secreted from BMSCs. Additionally, BMSCs exhibit potent modulation of neuroinflammation, by inhibiting monocyte infiltration, glial activation, and cytokine/chemokine production in the DRG and spinal cord. Thus, BMSCs control chronic pain by regulation of neuroinflammation in the PNS and CNS *via* paracrine signaling. In this review, we discuss the similar results from different laboratories of remarkable anti-nociceptive efficacy of BMSCs in animal and clinical studies. We also discuss the mechanisms by which BMSCs control neuroinflammation and chronic pain and how these cells specifically migrate to damaged tissues.

Keywords: neuroinflammation, bone marrow stem cells, chronic pain, treatment, transforming growth factor beta

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INTRODUCTION

Whereas acute pain can bring attention to the body of possible injuries and is normally a protective sensation, chronic pain does not convey any useful information and has no biological benefits. It only gives people a feeling of discomfort but does not play an active role in wound healing. Chronic pain can persist for months to years, even after the primary injury or inflammation has healed. Chronic pain is a major clinical problem that affects up to 30% of adults in the world (1, 2). Chronic pain costs the US economy more than $600 billion per year in healthcare expenditures, disability payments, and lost productivity (from American Pain Society) (2). Medications, massage therapy, acupuncture, electrical stimulation, nerve blocks, and surgery are some traditional therapies for chronic pain.

These methods can be powerful and effective for some patients. However, there are no drugs or treatments currently available that treat chronic pain in a complete and definitive way. Growing evidence suggests that bidirectional signaling between the immune system and the nervous system contributes to the development and maintenance of chronic pain (3, 4). Neuroinflammation results from the activation of glial cells in the peripheral nervous system including Schwann cells and satellite glial cells, in the central nervous system including microglia, astrocytes, and oligodendrocytes, as well as the activation of immune cells including resident mast cells and macrophages and infiltrating neutrophils and T cells.

The production of glial and pro-inflammatory mediators (e.g., cytokines, chemokines, trophic factors, neurotransmitters, and lipid mediators) modulates pain sensitivity, with persistent glial and immune cell activation and interaction with neurons leading to the development of peripheral and central sensitization, and induction of chronic pain conditions. In the periphery, an inflammatory milieu of interleukin (IL)-1β, TNFα, bradykinin, SP, CGRP, NGF, and prostaglandins is released by resident and infiltrating immune cells as well as from sensory nerve terminals (5). In the spinal cord, bidirectional signaling between neurons and glia regulated by chemokines (CXCL1, CCL2, and CX3CL1), proteases [metalloproteinase (MMP)-9, cathepsin S, and caspase 6], and the WNT signaling pathway involve in neuroinflammation and chronic pain sensitization (3). Thus, neuroinflammation is associated with various painful insults and pathologies which include neuropathic pain from nerve and spinal cord injury, inflammatory pain caused by arthritis, cancer pain, and pain caused by drug therapy.

Targeting the specific processes and molecules involved in neuroinflammation provides new therapeutic opportunities for chronic pain. For instance, inhibiting microglial response with minocycline and p38 MAP kinase inhibitor has been proven to prevent the initiation of neuropathic pain in rodents (6–8). Targeting activated astrocyte signaling through connexin-43 and CXCL1 inhibition has also demonstrated the ability to reverse established chronic neuropathic pain following peripheral nerve injury (5, 9). Pro-resolution lipid mediators (PRLMs) including resolvins, protectins, and lipoxins are new targets for treating chronic pain as they possess potent anti-inflammatory and anti-nociceptive properties through action on neurons, immune cells, and glial cells, but importantly do not suppress immune function. By protecting the body from bacterial and viral infection, PRLMs are poised as one of the most effective treatments for preventing surgical or trauma-induced chronic pain (3, 10).

BMSCs PRODUCE PAIN RELIEF IN ANIMAL MODELS

Recent studies have revealed the therapeutic potential of bone marrow stromal cells/bone mesenchymal stem cells (BMSCs) for chronic pain (11–14). BMSCs are a heterogeneous population of stromal cells present in bone marrow that give rise to various tissues throughout the body (15). Due to their strong immunosuppressive properties, BMSCs can be used in both autologous and heterologous transplantation without the need for immunesuppressive agents (16). BMSCs were originally considered by researchers as stem cells to reconstruct damaged and/or diseased tissues (15). However, recent studies have shown that BMSCs can affect a variety of physiological and pathophysiological processes, including immune and inflammatory responses, by releasing cytokines, chemokines, and trophic factors (17). Here, we review the immunomodulatory effects of BMSCs and the possible mechanisms of action. We also consider the implications of these data for clinical studies of BMSCs in the management of chronic pain.

The ability of BMSCs to alter the inflammatory milieu has made BMSCs an attractive treatment possibility for various painful states such as inflammatory pain, neuropathic pain, and cancer pain. Fortunately, many studies have reported pain relief in animal pain models with BMSC treatment (**Table 1**).

Inflammatory Pain

Osteoarthritis (OA) is a form of inflammatory pain with a significant impact on quality of life. At present only a few therapies are effective for OA patients, with most of them designed to relieve pain, control inflammation, and improve function (18). BMSC-based therapeutic efforts to treat OA have been well documented in different animal models including murine (19), rabbit (20, 21), sheep (22, 23), and horse (24). Autologous or allogeneic autologous BMSCs were injected into the joints of tested animals. Transplanted BMSCs were shown to reduce the progression of OA by controlling the inflammation, reducing cartilage loss, and improving cartilage content (25–27). Initially, BMSCs were considered the ideal source of direct regeneration of the articular surface. Recently, an increasing number of studies have shown that the major benefits of BMSCs come from paracrine activity (12, 25, 26, 28). Injections of MSCs have led to documented joint tissue regeneration; however, some studies have found that native cells are what primarily comprise reconstituted tissues with few transplanted cells contributing to regenerated tissue (23). Other studies have shown that the cell signaling milieu changes following delivery of MSCs specifically with a subsequent increase in host type II collagen production (19). Together, these factors suggest that MSCs may be coordinating a state of repair rather than directly replacing damaged tissues. This falls in line with the anti-inflammatory and immunomodulatory roles of MSCs.

Intervertebral disk degeneration is directly related to chronic inflammatory back pain. Cytokines in the degenerative tissue cause pain directly by enhancing protease activity (29). At present, there is no medication or treatment that can treat chronic back pain in a complete way without the risk of major side effects. Non-invasive therapies offer limited efficacy in the treatment of pain, and although surgical removal of the disk can relieve pain immediately, degeneration of adjacent segments can occur with the subsequent return of pain. In order to avoid recurrent pain and invasive surgical procedures, BMSC-based therapy is now being studied as a promising approach for the repair of degenerative intervertebral disks (30–32). In a similar role to their use in treating OA, BMSCs can also modulate the inflammatory microenvironment from intervertebral disk injury, and reduce inflammatory pain *via* paracrine pathways (31, 33, 34).

TABLE 1 | Pain relief by BMSCs under different injury and injection conditions.

Year	Reference	Disease (model) and species	Cell source	Number of cells	Delivery site	Effects on pain
2007	Musolino et al. (41)	SLNC, rat	Rat	2×10^5	Intraganglionic	Prevention of mechanical and thermal allodynia
2007	Klass et al. (50)	CCI, rat	Rat	1×10^7	Intravenous	Improvement of mechanical allodynia and thermal hyperalgesia
2008	Shibata et al. (45)	STZ-induced diabetes, rat	Rat	1×10^6	Injection in the hind limb skeletal muscle	Improvement of hypoalgesia
2009	Abrams et al. (42)	Spinal cord injury, rat	Rat	3×10^5	Injury site	Improvement of mechanical allodynia, no effect on thermal hyperalgesia
2010	Siniscalco et al. (44)	SNI, mouse	Human	5×10^4	Lateral cerebral ventricle	Improvement of mechanical allodynia and thermal hyperalgesia
2011	Siniscalco et al. (51)	SNI, mouse	Human	2×10^6	Intravenous	Improvement of mechanical allodynia and thermal hyperalgesia
2011	Orozco et al. (34)	Degenerative disk disease, human	Human	$10 \pm 5 \times 10^6$ per disk	Intradisc injection	Decrease in pain
2011	Guo et al. (11)	Chronic orofacial pain, rat	Rat	$1.5 \times 10^{3-6}$; $1.5{\sim}3.75 \times 10^5$	Intravenous; injury site	Reversed mechanical hypersensitivity
2011	Naruse et al. (46)	STZ-induced diabetes, rat	Rat	1×10^6	Injection in the hind limb skeletal muscle	Improves mechanical hyperalgesia, cold allodynia
2014	van Buul et al. (12)	Osteoarthritis, rat	Rat	1×10^6 per joint	Intra-articular injection	Decrease in pain
2014	Zhang, et al. (47)	SNL, rat	Rat	1×10^5	Intrathecal injection	Improvement of mechanical allodynia
2015	Chen et al. (14)	CCI, SNI, mouse	Mouse	$1{\sim}2.5 \times 10^5$	Intrathecal injection	Suppress neuropathic pain
2016	Pettine et al. (33)	Degenerative disk disease, human	Human	$2{-}4 \times 10^8$ nucleated cells per disk	Intradisc injection	Decrease in pain
2016	Yousefifard et al. (43)	Spinal cord injury, rat	Human	1×10^6	Injury site	Improvement of mechanical and cold allodynia; mechanical and thermal hyperalgesia
2016	Guo et al (52)	TL, SNL, CCI-ION, rat, and mice	Rat, human	1.5×10^6	Intravenous; injury site	Improvement of mechanical and thermal hyperalgesia; suppress aversive behavior
2017	Li et al. (48)	SNL, rat	Rat	2.5×10^6	Intrathecal injection	Improvement of mechanical allodynia and thermal hyperalgesia
2017	Fischer et al. (49)	TNI, rat	Rat	2.5×10^5	Intrathecal injection	Improvement of mechanical hyperalgesia

CCI, chronic constriction injury; SLNC, single ligature nerve constriction; SNI, spared nerve injury; SNL, spinal nerve ligation; STZ, streptozotocin; TL, tendon ligation; CCI-ION, chronic constriction injury of the infraorbital nerve; TNI, tibial nerve injury.

Inflammatory bowel disease (IBD) is marked by recurring and idiopathic intestinal inflammation, which can lead to significant morbidity and potential mortality. Symptoms include abdominal pain, cramps, bloody stool, and persistent diarrhea or constipation, all of which significantly impair a patient's quality of life (35). However, due to a lack of understandings of the origins of IBD, current treatment strategies fail to treat the root causes of IBD and mainly combat the symptoms of the disease. The treatments are further limited by a lack of efficacy as well as toxic and adverse side effect profiles (36). Recently, BMSCs have attracted increasing amounts of attention for the treatment of IBD (37–39). Intravenous injection of BMSCs in rats reduced the damage of the intestinal mucosal barrier, led to the down-regulation of zona occludens 1 expression, and reduced the intestinal damage mediated by a TNFα-mediated mechanism (40).

Neuropathic Pain

Neuropathic pain is a rather stubborn pain induced by nerve injury and can last for months to years, even after the primary tissue damage has healed. Current treatments for neuropathic pain are insufficient. However, in the context of neuropathic pain, transplantation of BMSCs has been shown to reduce the progress of neuropathic pain. In a variety of neuropathic pain models, BMSCs were injected directly into the lesion site [*via* intraganglionic (41), intraspinal (13, 42, 43), intrabrain (44), intramuscular (11, 45, 46), intrathecal injection (14, 47–49), or sytemic intravenous administration (11, 50–52)]. For example, intravenous injection of BMSCs reduced mechanical allodynia and thermal hyperalgesia in rodent chronic constriction injury (CCI) models of the sciatic (50) and infraorbital nerve (11, 52), as well as in spared nerve injury (SNI) models (51). Intramuscular injection of BMSCs reduced mechanical allodynia and cold pain in an STZ-induced diabetic model (45, 46) and also in an infraorbital nerve CCI model of orofacial pain (11). Intraspinal injection of BMSCs reduced mechanical allodynia and thermal hyperalgesia in spinal cord injury models of rats (42) and mice (13). Intraganglionic injections of BMSCs improved mechanical allodynia and thermal hyperalgesia in a rat single ligature nerve constriction model (41). Intrathecal injections of BMSCs improved mechanical allodynia and thermal hyperalgesia in a rat SNL model (47, 48).

In our recent paper (14), we demonstrated long-term analgesic relief of neuropathic pain in mice after a single intrathecal injection of BMSCs. Intrathecal administration of 250,000 BMSCs alleviated symptoms of early- and late-phase neuropathic

pain including allodynia and hyperalgesia, for several weeks in mice nerve injury models, including CCI and SNI. In addition, intrathecally administered BMSCs also alleviated CCI-induced ongoing pain. Furthermore, intrathecal BMSCs protected dorsal root ganglion (DRG) neurons from axonal injury and inhibited neuroinflammation in both DRGs and spinal cord tissues in CCI mice. Intrathecal BMSCs specifically target injured dorsal root ganglia *via* a CXCL12/CXCR4 interaction. Nerve injury-induced CXCL12 up-regulation in the injured L4–L5 DRGs leads to the trafficking of CXCR4-expressing BMSCs specifically to the injured DRGs. Intrathecal BMSCs inhibit neuropathic pain *via* transforming growth factor beta (TGF-β)1 secretion, which can be detected in the CSF. The analgesic effect of BMSCs was also reversed by TGF-β1 antibody but not by IL-10 antibody neutralization. Additionally, intrathecal injection of exogenous TGF-β1, at very low doses (1–10 ng) elicited potent analgesic effects in a neuropathic pain model. BMSCs can survive in DRGs for several months, without converting to other cell types, after which they disappeared from DRGs.

Cancer Pain

Cancer development is often associated with chronic inflammation (53). Although BMSCs have a potential anti-inflammatory effect, the exact role of BMSCs in tumor development remains controversial in the literature (54). Many studies have shown that BMSCs exhibit anti-tumor effects and inhibit tumor growth (55, 56), whereas other studies have suggested the role of pro-tumor effects (57, 58). In general, early-stage cancers often do not cause pain unless there are metastases to bone (59). To date, there is little literature showing the use of BMSCs to treat pain caused by the tumor itself. However, BMSCs have been found to treat chronic visceral pain induced by radiotherapy (60). Radiotherapy is a common type of cancer treatment. However, clinical studies have shown that about 50% of patients who receive radiotherapy have chronic visceral pain or tenesmus. Because of a cross-sensitization between visceral organs, cross sensitivity may amplify incoming signals and cause exacerbation of pain. Intravenously administered BMSCs 4 weeks following radiation treatments induced a time-dependent reversion of the visceral allodynia with a decrease in the anatomical interactions between mast cells and PGP9.5+ nerve fibers. Additionally, MSC treatment has the ability to limit colonic ulceration induced by radiation, a benefit that is not conferred by ketotifen (60).

ROUTE OF BMSCs ADMINISTRATION

Intrathecal administration is safe and there are several FDA-approved drugs for intrathecal injection. Several clinical trials show that intrathecal injection of BMSCs does not cause adverse health issues up to 12 months after treatment. Given the protection of the spinal/brain blood barrier, intrathecal administration of BMSCs can evade host immune responses. Thus, the intrathecal route can target a common pain pathway, produce long-term survival of BMSCs, and in turn provide long-term pain relief. In contrast, systemic administration such as through intravenous injection requires large numbers of BMSCs as most of the injected BMSCs will gather in the pulmonary capillaries and survive for a few days following injection (61–63). Furthermore, local BMSC injections, such as joint injections or intramuscular injections, can only target a single pain site and with short-term survival of BMSCs. Intraspinal or intraganglionic injections are very invasive procedures and may cause additional trauma which can further compromise injured tissue.

Several studies have shown how short-lived paracrine mechanisms are prominent amidst the various therapeutic actions of MSCs. Toma et al. (64) conducted a study where human MSCs (hMSCs) tagged with β-galactosidase were injected into the left ventricles of immunodeficient mice. Four days following injection, most of the injected hMSCs were found in the lung, spleen, and liver. Additionally only 0.44% of the injected hMSCs survived, but when compared with surrounding cardiomyocytes, these cells were morphologically indistinguishable. The findings from this study were reflected by other groups which have reported that less than 1% of systemically administered MSC cells survive for more than 1 week and that the secreted factors and not so much the cells themselves are responsible for the benefits of MSC therapy (62, 65).

MECHANISMS AND MEDIATORS BY WHICH BMSCs REDUCE PAIN AND NEUROINFLAMMATION

The mechanisms of the analgesic effects conferred by BMSCs are mainly due to the paracrine factors secreted by the cells (**Figure 1**). BMSCs produce a large amount of biologically active molecules which regulate different functions through the interaction of different cell types (66). Herein, we will describe some of the major factors secreted by BMSCs that associated with analgesic actions.

Transforming Growth Factor Beta

Transforming growth factor beta is a widely expressed secreted protein in various tissues that controls many cellular functions, including growth, proliferation, differentiation, and apoptosis. BMSC production of TGF-β has been shown by several investigators and immune modulation of BMSCs has been demonstrated to be partially mediated by TGF-β (14, 48, 61, 67). TGF-β is not only a powerful immunosuppressive cytokine but also a powerful neuromodulator. Previous studies have shown that BMSC administration can improve neurological function of animals with ischemic brain injury, with specific silencing of TGF-β abrogating the effects of the administered MSCs (68).

How does TGF-β1 reduce chronic pain? Accumulating evidence suggests that TGF-β1 inhibits nerve injury-induced activation and proliferation of microglia and astrocytes, and TGF-β1 also reduces the expression and secretion of pro-inflammatory cytokines (69–71). In our recent paper (14), we found that TGF-β1 can rapidly (within 1 min) modulate synaptic transmission in the spinal cord and neuronal excitability in DRG *via* a non-genomic mechanism. However, the mechanisms mediating TGF-β1 inhibition of neuropathic pain need to be further clarified. There is still controversy about the effects of TGF-β1 on chronic pain (72, 73). The results of different animal pain models are not consistent. In addition, TGF-β1 shows

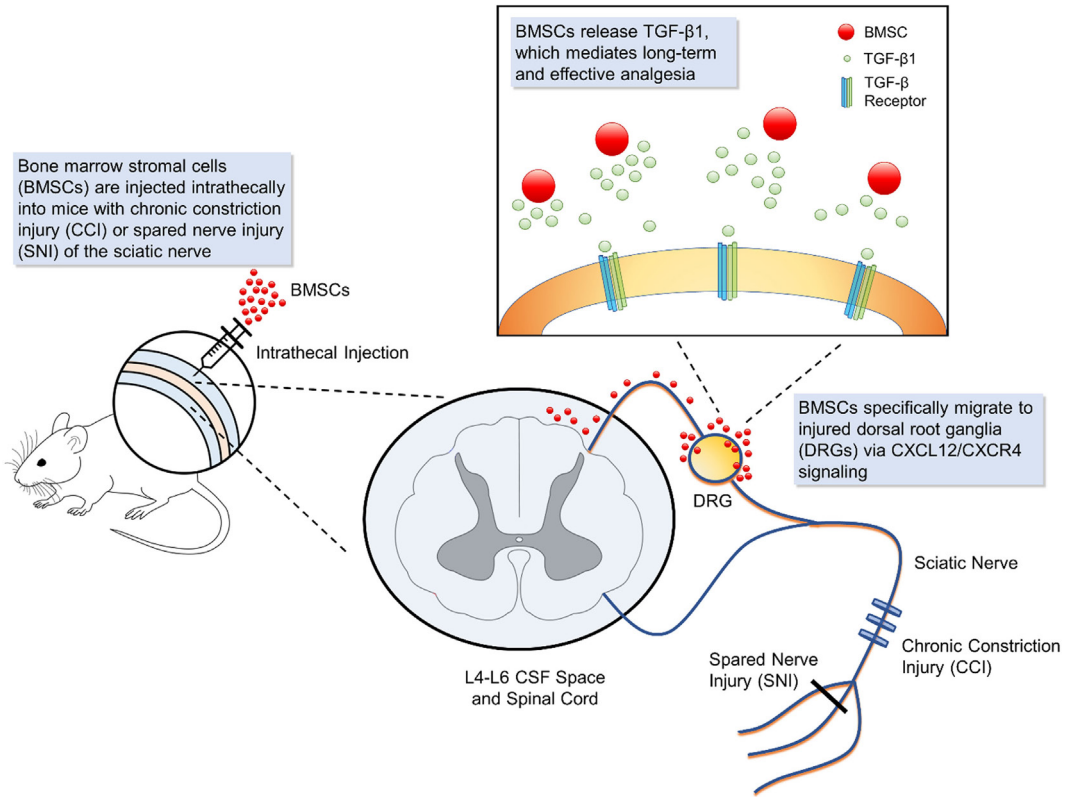

FIGURE 1 | Schematic of bone marrow stromal cell (BMSC) intrathecal injection for treating neuropathic pain. Intrathecal injection introduces bone marrow stromal cells into the cerebrospinal fluid. In mouse models of neuropathic injury of the sciatic nerve, including chronic constriction injury and spared nerve injury, BMSCs expressing CXCR4 specifically migrate to the L4-L6 dorsal root ganglia (DRG) where injured neurons up-regulate the corresponding ligand CXCL12. At the DRG, BMSCs secrete transforming growth factor beta 1, a powerful neuromodulator which rapidly suppresses spinal synaptic plasticity, DRG neuronal hyper-excitability, and neuropathic pain resulting from neuropathic injury. BMSCs that migrate to injured DRGs have been found to survive for 2 months, providing effective, and sustained analgesia.

different activities on different cell types and even a single cell type at different stages of development. Identifying the details of the TGF-β1 analgesic signaling pathway will be important in developing our understanding of how TGF-β1 expression leads to analgesia in chronic pain conditions.

Interleukin-10

Interleukin-10 is a powerful anti-inflammatory cytokine with multiple effects in immunoregulation and inflammation. The role of IL-10 in reducing chronic pain has been recognized and the secretion of IL-10 by BMSCs was investigated in several studies (48, 74, 75). Compared with high production of TGF-β, IL-10 release from BMSCs was very low (14). The role of IL-10 in BMSC-mediated analgesia remains controversial. Some authors have reported no significant changes in the level of IL-10 when using BMSCs and BMSC-conditioned media in different models *ex vivo* and *in vitro*. We found that IL-10 release from BMSCs did not contribute to BMSC-induced pain relief. The analgesic effect of BMSCs was neutralized by TGF-β1 antibody, but not IL-10 antibody (14). However, a recent study showed that the analgesic effects of intrathecally injected BMSCs were reversed by both TGF-β1 and IL-10 antibody neutralization (48).

Tumor Necrosis Factor-Stimulated Gene-6 (TSG-6)

Tumor necrosis factor-stimulated gene-6 is a glycoprotein that was shown to produce potent anti-inflammatory effects. The relationship between BMSCs and TSG-6 has been intensively investigated (76). Therapeutic effects of BMSCs in some animal models of disease, such as cerebral ischemia (77), diabetes type 1 (78), peritoneal adhesions (79, 80), and experimental autoimmune encephalomyelitis (EAE) (81), was observed to be dependent on TSG-6. Cell tracking studies demonstrated that intravenously administered BMSCs mostly were trapped in the lung. Microarray analysis of BMSCs found in the lung revealed that one of the highest up-regulated transcripts was TSG-6. The silencing TSG-6 in BMSCs prior to administration resulted in loss of their therapeutic properties, while exogenous TSG-6 administration actually replicated the therapeutic activity provided by BMSCs (79).

Hepatocyte Growth Factor 1 (HGF-1)

Hepatocyte growth factor 1 is a paracrine cellular growth, motility, and morphogenic factor, with a number of regenerative processes linked to its activation (82). Like other soluble factors that

are implicated in regenerative processes, HGF-1 demonstrates immune modulatory activity. *In vivo* administration of HGF-1 has been shown to provide a protective effect from autoimmune disease, *via* activating regulatory T cells which produce the immunosuppressive cytokine IL-10 (83, 84). One of the key mediators responsible for the therapeutic activities of BMSCs *in vivo* is the production of HGF-1. When antibodies blocking HGF-1 are introduced, they cancel the protective effects of BMSCs and BMSC-conditioned media in the EAE model of multiple sclerosis (85). HGF-1 also appears to be necessary for the neuroprotective effects of MSC-conditioned media, demonstrated by experiments where apoptotic processes were not protected against in a glutamate-induced excitotoxicity model when HGF-1 function was neutralized (86). The HGF-1 generated from MSCs therefore shows a number of different effects, including angiogenesis, immune modulation, and protection from apoptosis.

Metalloproteinases

Recently, human umbilical cord plasma was found to be enriched with tissue inhibitor of metalloproteinases 2 (TIMP-2) in a study that showed how systemic treatments of umbilical cord plasma and TIMP-2 increased synaptic plasticity and hippocampal-dependent cognition in aged mice (87). MMPs have been shown to play major roles in neuroinflammation and pain. MMP-9 produced by injured DRG neurons leads to the development of early-phase neuropathic pain through the activation of microglia, IL-1β cleavage, and microglial p38 activation. MMP-2 production leads to late-phase neuropathic pain caused by IL-1β cleavage and astrocytic ERK activation. TIMP proteins suppress neuropathic pain, with TIMP-1 alleviating early-phase neuropathic pain and TIMP-2 attenuating established late-phase neuropathic pain (88). The presence of TIMP-2 in umbilical cord plasma suggests the possibility that similar adult mesenchymal stem cell populations such as BMSCs may produce TIMP proteins which can likewise inhibit MMP-mediated neuroinflammation and pain.

BMSCs IN CLINICAL PRACTICE FOR PAIN MANAGEMENT

Transplantation of BMSCs is considered safe and has been extensively tested in clinical trials, including cardiovascular, neurological, and immunological disease, with exciting results. The growing interest in MSC therapy stems from their safety in treatments as well as the pleiotropic functions of MSCs that enhance endogenous repair mechanisms and attenuate immunological dysfunction. Currently, there are over 200 registered clinical trials sites worldwide for the evaluation of MSC treatment [http://clinicaltrials.gov/, summarized in Ref. (38)]. Investigators have also recognized the potential for BMSCs to treat painful diseases in patients (**Table 1**).

A pilot study (34) had 10 patients with degenerative disk disease and lower back pain receive autologous BMSCs. These patients reported a decrease in pain and disability at a level that was comparable with patients who received spinal fusion or total disk replacement surgery. Additionally, BMSC therapy offered the advantages of being a less invasive procedure and that helped to preserve the biomechanical functions of the lumbar spine. A study from Japan showed that degenerated intervertebral disks that received injections of autologous MSCs led to disk regeneration as well as reported alleviation of back and leg pain (89). In 2008, Centeno et al. discovered that the meniscus cartilage in a knee joint of an OA patient showed regeneration after intra-articular implantation of autologous MSCs (90). Subsequent safety reports for the use of MSCs in treating OA by Centeno's group showed that follow-up MRIs in 227 patients at various time points ranging from 3 months to 2 years following implantation of BMSCs revealed no new tumor formations at the re-implantation sites (91).

To provide salvage treatment for cancer patients who received overdoses of radiotherapy, intravenous administration of allogeneic BMSCs from relatives of patients was performed in three patients with refractory and fistulizing colitis resembling fistulizing Crohn's disease (37) and achieved a therapeutic effect. Systemic BMSC therapy of refractory irradiation-induced colitis is considered a safe and effective treatment for relieving symptoms, such as pain, inflammation, diarrhea, and hemorrhage. BMSCs also regulate immune response through an increase of T regulatory cells and a decrease of activated effector T cells (92). Encouraging results have been observed from clinical trials of Crohn's disease, systemic lupus erythematosus, and systemic sclerosis (37, 38, 93). These data indicate that BMSCs offer a promising therapy strategy for a variety of immune-related diseases.

PERSPECTIVE

To date, the possible mechanisms of the analgesic effects mediated by BMSCs and their associated paracrine factors are still not very clear. Different types of growth factors, anti-apoptotic factors, anti-inflammatory and pro-inflammatory factors, and chemoattractants cause these effects. As more studies focus on each specific cytokine released by BMSCs, it has been demonstrated that blocking at least one of them leads to a decrease in the therapeutic effect. BMSCs seem to produce a complex network of cytokines, which can be found in BMSC-CM. This is why the effect of BMSC-CM is often similar to the effect of BMSCs themselves. BMSCs and BMSC-CM have clearly demonstrated measurable therapeutic effects in different models of acute renal, lung, hepatic, ischemia–reperfusion, and burn injury. It is important to continue these studies for further implementation of this therapy in clinical practice.

Recently, the paracrine functions of BMSCs have been found to be mediated, at least in part, by extracellular vesicles (EVs) (94, 95). EVs are mainly secreted from the endosomal compartment and contain contents from their cells of origin, such as miRNA, mRNA, and proteins (96, 97). Recently, animal model-based studies have shown that EVs have important potential as a novel advancement over whole cell therapies (98, 99). It is also important and necessary to understand the contents of EVs, the mechanism of EV exocytosis, and the therapeutic effects of EVs.

Unexpectedly, there are gender-dependent differences in the secretion of different cytokines by BMSCs *in vivo*. Crisostomo et al. (100) found that in BMSCs from female mice stimulated

with LPS or hypoxia, the secretion level of VEGF was higher and the levels of TNF and IL-6 were significantly lower than in BMSCs from male mice. Additionally, in female mice, pro-inflammatory activity was lower while proliferative and regenerative activity was higher compared with male mice. Perhaps estrogen plays a role in these differences. It has been demonstrated that exogenous estrogen can increase the activity of MSCs and that MSCs express an estrogen receptor alpha, which may have played an important role in the proliferation and differentiation of MSCs (101). Further studies will be important to check for sex-dependent analgesic effects and signaling of BMSCs in chronic pain treatment.

CONCLUSION

An increasing number of articles are being published that describe the anti-inflammatory effects attributed to BMSCs and their paracrine factors. With the association of neuroinflammation and various painful insults and pathologies, these studies demonstrate the ability of BMSCs to treat chronic pain. Due to their strong immunoregulatory properties and high expansion potential, BMSCs can be used for successful autologous and even heterologous transplantation. Therefore, injections of BMSCs may provide efficient, long-term, and safe therapy for patients with painful diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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CCL5–Glutamate Cross-Talk in Astrocyte-Neuron Communication in Multiple Sclerosis

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The immune system (IS) and the central nervous system (CNS) are functionally coupled, and a large number of endogenous molecules (i.e., the chemokines for the IS and the classic neurotransmitters for the CNS) are shared in common between the two systems. These interactions are key elements for the elucidation of the pathogenesis of central inflammatory diseases. In recent years, evidence has been provided supporting the role of chemokines as modulators of central neurotransmission. It is the case of the chemokines CCL2 and CXCL12 that control pre- and/or post-synaptically the chemical transmission. This article aims to review the functional cross-talk linking another endogenous pro-inflammatory factor released by glial cells, i.e., the chemokine Regulated upon Activation Normal T-cell Expressed and Secreted (CCL5) and the principal neurotransmitter in CNS (i.e., glutamate) in physiological and pathological conditions. In particular, the review discusses preclinical data concerning the role of CCL5 as a modulator of central glutamatergic transmission in healthy and demyelinating disorders. The CCL5-mediated control of glutamate release at chemical synapses could be relevant either to the onset of psychiatric symptoms that often accompany the development of multiple sclerosis (MS), but also it might indirectly give a rationale for the progression of inflammation and demyelination. The impact of disease-modifying therapies for the cure of MS on the endogenous availability of CCL5 in CNS will be also summarized. We apologize in advance for omission in our coverage of the existing literature.

Keywords: CCL5, glutamate, nerve endings, multiple sclerosis, experimental autoimmune encephalomyelitis mice, release

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INTRODUCTION

The immune system and the central nervous system (CNS) cross-talk, and this interaction are pivotal to the onset and the progression of central neurodegenerative diseases (i.e., Alzheimer's disease and amyotrophic lateral sclerosis), as well as in classic autoimmune-inflammatory disorders [i.e., multiple sclerosis (MS)]. Although inflammation probably does not represent an initiating factor, new evidence suggests that pro-inflammatory molecules contribute to the derangement of chemical synapses favoring disease progression (1). In fact, a number of endogenous molecules (i.e., cytokines and chemokines) released by the immunocompetent cells as well as by activated astrocytes control chemical transmission at active synapses, affecting the main functions of these cells including

transmitter release and second messenger production. These endogenous molecules are main transducers of the pathological "glial to neuron" cross-talk (1–5).

Chemokines: General Considerations

In mammals, the chemokine (chemotactic cytokines) universe comprised of approximately 50 endogenous small (8–14 kDa) peptides released by immune cells and 20 receptors (4, 5). Chemokines have a tertiary structure highly conserved and are subdivided into four groups (namely the CC, the CXC, the CX_3C, and the C subfamilies) based on the relative positions of two conserved cysteine residues near the N-terminus (6). Chemokines act at chemokine receptors that are seven transmembrane domain *Pertussis* toxin (PTx)-sensitive, G-protein-coupled receptors (GPCRs), which depending on the G protein involved, trigger enzymatic cascade of events controlling several intraterminal pathways, mostly controlling Ca^{2+} ions mobilization, intraterminal phosphorylative pathways, and small Rho GTPases signaling (7). First identified for their ability to mediate leukocyte chemo-attraction in inflammatory and autoimmune diseases (8), chemokines and their receptors are now recognized as a promiscuous and redundant system of signaling interactions and mutual binding relevant to inflammation, immunity and neuropathology. Most of the chemokine receptors bind more than one ligand, and several chemokines activate more than one receptor, accounting for the numerical mismatch among chemokines and relative receptors. In particular, CCR1, CCR3, and CCR5 are promiscuous receptors for different chemokines including CCL3 (macrophage inflammatory protein 1-alpha), CCL5 [Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES)], and CCL7 (monocyte chemotactic protein-3). All these aspects have been largely documented in previous articles (3, 4, 9–19) and will not be further detailed.

CCL5

CCL5 plays a main role in inflammatory diseases and in cancer, because of its ability to control the movements of memory T lymphocytes, monocytes macrophages, and eosinophils (4–7). Evidence has been provided also showing a role of this chemokine in CNS diseases secondary to viral infections, such as the acquired immuno deficiency syndrome-related dementia, or involving neuro-inflammatory processes, such as MS, Alzheimer dementia, and Parkinson's disease (6, 12, 13, 20–23).

CCL5 is a 68-amino-acid protein that binds both pertussis toxin (PTx)-sensitive GPCRs (6, 8, 12) and Ptx-insensitive GPCRs (24) in the CNS. As to the central role of CCL5, the chemokine controls positively the mobilization of cytosolic Ca^{2+} and second messenger production in cultured neurons (25–28), astrocytes (29), and microglia (30), but it also activate GPCRs negatively coupled to adenylyl cyclase (AC)-mediated signaling, leading to the reduction of the endogenous level of cytosolic cyclic adenosine monophosphate (cAMP) (**Figure 1**) (25, 29).

CCL5 PRODUCTION IN CNS

The endogenous level of CCL5 is very low, almost undetectable, in cerebral spinal fluid (CSF) of healthy individuals, but it increases

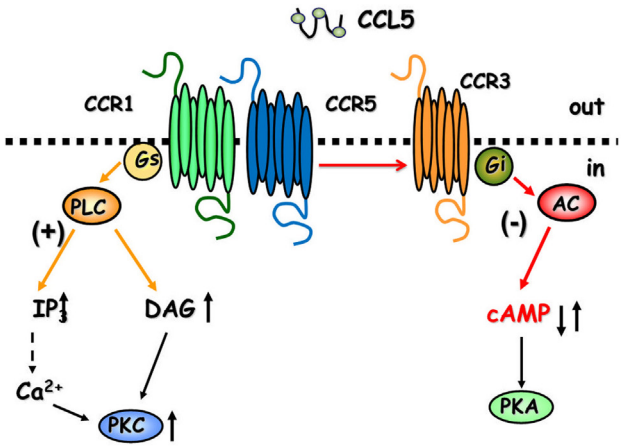

FIGURE 1 | The cartoon summarizes the intraterminal pathways involved in the facilitation and in the inhibition of glutamate exocytosis in mammals glutamatergic nerve endings. CCR1/CCR5 heterodimers couple to G proteins leading to phospholipase C (PLC) translocation, hydrolysis of membrane phosphoinositides, and production of inositol triphosphate (IP_3) and diacyl glycerol (DAG), which in turn mobilize Ca^{2+} ions and activate phosphorylative processes that favor vesicle exocytosis. The involvement of CCR3 in the heteromeric assembly of CCRs favors the coupling to inhibitory G proteins reducing adenylyl cyclase (AC) activity and low cyclic adenosine monophosphate (cAMP) production. These events account for reduced vesicular exocytosis.

dramatically when human immunodeficiency virus 1 (HIV-1) infection occurs (31–35), at the onset and during the progression of MS (9, 36–40). Increased central and peripheral CCL5 levels are also detected in mice suffering from the experimental autoimmune encephalomyelitis (EAE), an animal model reproducing most of the spinal cord pathological features of MS in humans [(41) and references therein]. Two are the mechanisms determining the dramatic increase of CCL5 bioavailability in CNS. First, the permeabilization of the blood–brain barrier that occurs in inflammation favors the entry of CCL5 from the periphery into the brain. Second, the concomitant massive local production of CCL5 from astrocytes and, to a lesser extent, from microglial cells triggered by pro-inflammatory citokines.

Microglia cells coordinate brain innate immunity, rapidly expand their population, and then migrate chemotactically to sustain inflammation and cells death. Microglia exist in the "resting" and the "activated" forms. In the resting state, that is an active surveying state, microglia cells in close proximity to neurons and astrocytes participate to the central network by releasing regulatory agents and by controlling homeostasis. In the "active" state, microglia releases pro-inflammatory effectors including TNF-alpha and IL-1beta, which diffuse to neighboring astrocytes, influencing their functions (42). These factors are the principal inducers of chemokine overproductions from astrocytes. Microglia filopodia make dynamic contact with astrocytes and neurons and have a pivotal role in controlling synaptic plasticity (43–45). The intimate contact of microglia with chemical synapses also makes the neuronal control of microglial functions possible. Actually, microglia cells are endowed with receptors for transmitters (46) the activation of which controls their activation

state. Excitatory transmission favors the shift from the "resting" to the "activated" state of microglial cells, while inhibitory transmission reduces activation of microglial cells to the "resting" condition (47).

Astrocytes are innate immune sentinels that ensheath cerebral blood vessels controlling the entry of peripheral cells into the CNS parenchyma during infection. Astrocytoma cells as well as cultured astrocytes produce CCL5 upon incubation with the microglial effectors TNF-alpha or IL-1 beta, through an inflammatory-like response mediated by the p55 receptor-dependent signaling (42, 48). IFN-gamma from microglial cells, inactive on its own (42), seriously amplifies the effect of either TNF-alpha or IL-1 beta, worsening the pro-inflammatory signaling.

The expression and the release of CCL5 from astrocytes is tightly controlled by several receptor subtypes, including opioid receptors (49, 50), group III metabotropic glutamate receptors (2), alpha/beta noradrenergic receptors (51–53), and sphingosine-1-phosphate receptor (S1PR) subtype 1 (54), as well as by endogenous neurotoxin such as quinolinic acid acting at NMDA receptors (55). These receptors represent targets of therapeutics for inflammatory autoimmune disease typified by overexpression of CCL5.

Differently, data supporting the active production and release of the endogenous chemokine from neurons are lacking, suggesting that these cells do not release the chemokine.

EXPRESSION OF THE CHEMOKINE RECEPTORS TARGETED BY CCL5 IN GLIAL CELLS

Once released in the synaptic cleft, CCL5 regulates the function of glial cells (microglia and astrocytes) themselves through autocrine processes.

The autocrine control of microglia functions is permitted by the existence of CCR5 receptors, and, to a less extent, of CCR1 and CCR3 receptors in these cells, which also permit the chemotactic movements of microglial cells during inflammation (56). CCR5s are also the receptors functional to HIV-1-induced pathogenetic mechanisms (57–59). As to the astrocytes, the existence of CCR1, CCR3, and CCR5 proteins in these cells has been a matter of discussion, and, despite some initial discrepancies (60), it was definitively demonstrated that adult astrocytes possess CCR1, CCR3, and CCR5 (61–63). CCR1, CCR3, and CCR5 proteins are expressed in cultured fetal astrocytes and in adult astrocytes from mammalian brains [(29), but see for a concise review Ref. (60)], as well as in astrocytic processes [gliosomes (64, 65)] isolated from the cortex and the spinal cord of adult mice (**Table 1**) (66). In particular, in physiological conditions, the expression of CCR5 in astrocytes is low but rapidly increases to abnormal pathological levels upon stimulation with TNF-alpha and by IL-1beta released by neighboring microglial cells, which causes persistent adaptation leading to a significant increase in the expression of most of the CCR proteins (55). This cascade of events occurs during inflammation, so that the overexpression of the receptors targeted by CCL5 is an event intimately linked to pathological conditions.

CCL5 AND GLUTAMATE RELEASE FROM ASTROCYTES

In cultured astrocytes from both fetal simian and human brains, a significant increase in internal Ca^{2+} ions mobilization was observed upon exposure to CCL5 (29). Since intraterminal Ca^{2+} ions' mobilization is a prerequisite to transmitter exocytosis also in these cells [(77) and references therein] the CCL5-mediated control of cytosolic Ca^{2+} bioavailability might suggest that the chemokine could favor/modulate glutamate exocytosis from astrocytes. Despite the expectation, however, the spontaneous and the depolarization-evoked release of [^{3}H]D-aspartate ([^{3}H]D-Asp) from gliosomal particles isolated from human brain tissue was not modified by CCL5 (29), compatible with the idea that activation of these receptors cannot modify the glutamate outflow of the excitatory aminoacid. Similarly, exposure of human and mouse glial particles [we refer to as gliosomes (64)] to CCL5 in the absence or in the presence of a concomitant depolarizing stimulus (i.e., 20 mM KCl-enriched solution, KCl substituting for an equimolar amount of NaCl) did not significantly modify the outflow of glutamate. This is compatible with the idea that the amount of Ca^{2+} ions mobilized in gliosomal cytosol by CCL5 acting at its own receptors was insufficient to prime vesicle docking and fusion with astrocyte membranes (66, 71). So far, data concerning the impact of CCL5 on glutamate uptake in microglia and astrocytes are not available and further investigations are needed to address these aspects.

CCL5 IN NEURONS

Expression of Chemokine Receptors Targeted by CCL5 in Neurons

Once released in the biophase, CCL5 also reaches neurons through a mechanism of volume diffusion, to modulate neuronal functions via paracrine mechanisms, mediated by CCRs express in neurons. This regulation eventually occurs when the external concentration of the chemokine is high and assures a sufficient diffusion of the agent in the synaptic space.

The first report of the existence of chemokine receptors in neurons dates to 1997 [(67), **Table 1**]. In this article, by combining immune-histochemical staining with receptor binding studies, the authors demonstrated that cultured human neuronal cells express CXCR2, CXCR4, CCR1, and CCR5 receptors. The authors suggested that these entities represent the binding site for the viral envelope protein gp120 of the HIV-1 virus on neuronal plasmamembranes, allowing a CD4-independent interaction between the virus and the neurons. Soon after, in 1998, Meucci et al. (25) demonstrated that cultured hippocampal neurons are endowed with several chemokine receptors, including CCR1 and CCR5 subtypes. In line with these observations, in1999, Klein et al. (29) provided clear evidence that a subpopulation of neurons in the cortex of human and macaque brains are endowed with CCR3, CCR5, and CXCR4 receptors. The existence of CCR1, CCR3, and CCR5 receptor proteins in neurons was then confirmed by other groups (20, 78–81), despite some discrepancies concerning their exact location (in the soma, on axonal processes and/or in

TABLE 1 | Distribution of CCR1, CCR3, and CCR5 in astrocytes and neurons in the central nervous system (CNS) of mammals.

	Cell types	CNS region	Species	mRNA	Protein	Reference
CCR1	Neurons	Cultured neurons	Human	+		(67)
		Fetal brain	Macacque			(68)
		Cerebellum	Rat			(27)
		Hippocampus	Rat	+		(25)
		Neonatal and adult cerebellum	Rat	+		(69)
		Neonatal and adult cerebellum	Rat	+		(70)
		Cortical nerve endings	Human			(71)
		Cortical and spinal cord nerve endings	Mouse			(66, 72)
	Astrocytes	Neonatal brain	Mouse	+		(73)
		Neonatal brain	Mouse	+	+	(74)
		Fetal brain	Human		+	(75)
		Neonatal and adult cerebellum	Rat	+	+	(69)
		Neonatal and adult cerebellum	Rat	+	+	(70)
		Spinal cord gliosomes	Mouse		+	(66)
CCR3	Neurons	Cerebellar neurons	Rat		+	(27)
		Cortical neurons	Fetal human		+	(29)
			Fetal macaque		+	(29)
		Cortical nerve endings	Human		+	(71)
		Cortical and spinal cord nerve endings	Mouse		+	(66, 72)
		Fetal brain neurons	Human		+	(20)
	Astrocytes	Primary astrocytes	Human	+	+	(62)
		Fetal and adult astrocytes	Human, macaque	+	+	(76)
		Spinal cord gliosomes	Mouse		+	(66)
		Fetal brain astrocytes	Human		+	(20)
CCR5	Neurons	Neonatal DRG	Rat	+		(68)
		Cerebellar neurons	Rat		+	(27)
		Cortical neurons	Fetal human		+	(29)
			Fetal macaque		+	(29)
		Hippocampal neurons	Rat	+		(25)
		Embryonic neurons	Human	+		(26)
		Cortical nerve endings	Human		+	(71)
		Cortical and spinal cord nerve endings	Mouse		+	(66, 72)
		Neonatal brain	Rat	+	+	(48)
		Brain	Human		+	(33)
	Astrocytes	Cortical astrocytes	Fetal human		+	(29)
			Fetal macaque		+	(29)
		Fetal brain neurons	Human		+	(20)
		Fetal and adult astrocytes	Human		+	(76)
		Spinal cord gliosomes	Mouse		+	(66)
		Neonatal brain	Rat	+	+	(48)
		Brain	Human		+	(33)

nerve terminals). As to this aspect, a relevant finding was that CCR1, CCR3, and CCR5 receptor proteins exist in both human and rodent cortical nerve endings, as well as in rodent spinal cord terminals, i.e., in those parts of neuron where transmitter exocytosis occurs (66, 71, 72).

CCL5 and Glutamate Release from Neurons

As observed in astrocytes, CCL5 controls the movement of Ca^{2+} ions in neurons (25, 29) but differently from astrocytes, this effect is sufficient to trigger changes in glutamate release efficiency. Based on these first observations, as well as on data published about a decade later (66, 71), the impact of CCL5 on glutamate release was found to represent a complex event, strictly dependent on the activity of the neurons themselves and on the region of the CNS under study.

When studying the changes in Ca^{2+} cytosolic bioavailability in hippocampal cultured neurons, Meucci et al. (25) showed that nanomolar CCL5 favored Ca^{2+} ion mobilization in resting condition but significantly reduced the increase in cytosolic Ca^{2+} that follows exposure of neurons to a depolarizing stimulus. Similarly, low nanomolar concentrations of the human recombinant CCL5 (hCCL5) exert opposite control on glutamate release [measured as the release of the unmetabolizable marker of glutamate, the compound [^{3}H]D-Asp (82–85)] from nerve endings (synaptosomes) isolated from cortical specimens that were removed from consenting patients undergoing neurosurgery to reach deeply located tumors (71). In particular, hCCL5 elicited a significant increase in the spontaneous release of [^{3}H]D-Asp from these

terminals in basal conditions (i.e., in the absence of a depolarizing stimulus).

Facilitation of glutamate outflow relied on the activation of PTx-sensitive GPCRs positively coupled to phospholipase C (PLC)-mediated events, the activation of which led to the hydrolysis of membranes phosphoinositide and the mobilization of Ca^{2+} ions from Xestospongin-C-sensitive, inositol triphosphate (IP_3)-dependent intraterminal stores located in the endoplasmic reticulum. Facilitation of glutamate release, however, turned to inhibition when hCCL5 was applied concomitantly to a mild depolarizing stimulus (i.e., 12 mM KCl). In this case, inhibition relied on the binding of hCCL5 to PTx-sensitive GPCRs negatively coupled to the AC/cAMP/protein kinase A (PKA) intraterminal enzymatic pathway (**Figure 1**). Comparable results were obtained when studying the impact of hCCL5 on human cortical slices. hCCL5 increased the basal release of [^{3}H]D-Asp but significantly reduced the tritium overflow elicited by depolarizing stimuli (71).

Human recombinant CCL5-mediated facilitation of the spontaneous outflow of glutamate from both isolated nerve terminals and slices was prevented by MetRANTES, a broad-spectrum antagonist of the CCR1, CCR3, and CCR5 subtypes, confirming the involvement of these receptors (71). The impacts of the chemokine in the different experimental conditions (resting versus depolarized condition), however, seemed predictive of the existence of receptor subtype oligomers. Since antagonists able to discriminate among the different CCR subtypes were not available at that time, the pharmacological characterization of the receptor(s) accounting for the hCCL5-induced changes of glutamate outflow was carried out by pre-incubating human synaptosomes with antibodies raised against the N-terminal of the CCR1, the CCR3, and the CCR5 receptor proteins. By binding selectively to the outer side of the receptor protein, antibodies are expected to impede the interaction of the agonist with the orthosteric binding site, then mimicking receptor antagonists (86, 87). Pre-incubation of synaptosomes with antibodies raised against the extracellular NH_2 terminals of CCR1 or of CCR5 receptor proteins impeded the hCCL5-induced facilitation of glutamate outflow from cortical nerve endings, while pre-treatment with anti-CCR3 was ineffective. Differently, hCCL5–mediated inhibition of glutamate exocytosis was prevented by pre-incubating synaptosomes with anti-CCR1, anti-CCR3 or anti-CCR5 antibodies, consistent with the view that different CCR oligomers account for the opposite effects observed.

The main criticism to the results obtained with human nerve terminals concerned the potential confounding factors originating from the origin of the human specimens, i.e., the brain of patients suffering from cerebral tumors. The receptor repertoire involved in the CCL5-mediated control of glutamate exocytosis in human specimens could have been altered because of the pathological overexpression of CCL5 in glioma cells. The effects of hCCL5 in human cortical synaptosomes, however, were soon after reproduced in glutamatergic nerve endings isolated from the cortex of mice, which represent healthy individuals, where the endogenous CCL5 level is expected to be low (88). Again, in mouse cortical terminals, the release of glutamate in basal condition (i.e., the absence of a depolarizing stimulus) was potentiated by CCL5, but the chemokine significantly inhibited the glutamate exocytosis evoked by a mild K^+ depolarization (12 mM KCl) stimulus. The comparable effects observed in human and mice terminals allowed to conclude that the effects observed in human nerve endings were not influenced by the pathological origin of the tissue specimens.

Facilitation of the spontaneous release of glutamate as well as inhibition of 12 mM K^+-evoked glutamate exocytosis from cortical synaptosomes was prevented by the selective CCR1 antagonist BX513 and by the selective CCR5 antagonist DAPTA, compatible with the involvement of CCR1/CCR5 heterodimers in the effect observed (66). Furthermore, the CCR3 antagonist, the compound SB 328437, failed to affect the CCL5-mediated facilitation of glutamate release in basal condition, but it strongly prevented the inhibitory effect exerted by the chemokine in depolarized nerve terminals. Comparable results could be drawn when using antibodies raised against the N-terminal of the CCR1, CCR3, and CCR5 receptor proteins, leading to conclude that (i) the receptor composition of the chemokine oligomers controlling glutamate release in mouse and human cortical nerve endings is largely conserved, (ii) the involvement of CCR3 in the oligomer expression dictates the coupling to inhibitory G proteins bridging negatively the chemokine receptor complex to the AC/cAMP/PKA transducing mechanism (**Figure 1**; **Table 2**), and (iii) the mouse brain tissue is appropriate to investigate the effects of CCL5 on central glutamatergic transmission (66).

CCL5-mediated control of glutamate release in nerve terminals was not restricted to the cortex. The chemokine also efficiently modulates the release of glutamate from spinal cord nerve endings. However, differently from what observed in the cortex of adult mice, the spontaneous release of glutamate from spinal cord glutamatergic nerve endings was unaffected by nanomolar CCL5, while the depolarization-evoked glutamate exocytosis was significantly increased. Again, facilitation of glutamate

TABLE 2 | Correlation between the composition of CCR oligomers and the CCL5-mediated changes to glutamate release.

	Human cortical synaptosomes		Mouse cortical synaptosomes		Mouse spinal cord synaptosomes	
	Basal glutamate release	**12 mM KCl-evoked glutamate overflow**	**Basal glutamate release**	**12 mM KCl-evoked glutamate overflow**	**Basal glutamate release**	**15 mM KCl-evoked glutamate overflow**
CCR1	↑	↓	↑	↓	↑	↑
CCR3	Not involved	↓	Not involved	↓	Not involved	Not involved
CCR5	↑	↓	↑	↓	↑	↑

The table resumes the modulatory action of CCL5 on the release of glutamate (measured as release of preloaded [^{3}H]D-aspartate) correlating these events to the CCR subunits involved in the expression of chemokine oligomers targeted by the chemokine.

↓, inhibition of glutamet relesae; ↑, facilitation of glutamate exocytos.

exocytosis from these terminals involved CCR1/CCR5 heteromers positively coupled to PLC-induced IP_3-mediated enzymatic pathway, leading to increased mobilization of Ca^{2+} ions in the cytosol. Also in this region, the receptor protein composition was clarified by using selective CCR antagonists as well as anti CCR antibodies recognizing the N-terminal of the receptor protein (66). These studies unveiled that CCR1/CCR5 heterodimers mediates the CCL5-induced facilitation of glutamate exocytosis, further confirming the hypothesis that CCR1/CCR5 oligomers preferentially couples to stimulatory G protein positively coupled to PLC-mediated events (**Figure 1**; **Table 2**). Notably, CCR3 immunoreactivity was detected in spinal cord synaptosomal lysates, but this receptor subtype was not involved in the CCL5-mediated effect.

CCL5 IN DEMYELINATING DISORDERS

The serum level of CCL5 was found to be significantly increased in patients suffering from MS (89–91) as well as in EAE animals (15, 41, 92). The highest levels of the chemokine were detected in the peripheral blood mononuclear cells (PBMCs) of MS patients suffering from the secondary progressive form of the disease, while lower level was observed in the PBMCs from patients with the relapsing–remitting form of MS (93). CCL5 levels are also increased in the CNS of MS patients as well as of EAE mice (9, 13, 22, 88, 93–100). The huge elevation of the CCL5 levels in CNS mainly depends on the increased peripheral production of the chemokine, but it also reflects the local overexpression of the chemokine in astrocytes activated by IL-1beta, TNF-alpha, and IFN-gamma (55), from neighboring microglia cells. All these observations are predictive of the role of CCL5 in the onset and progression of disease in MS patients. Accordingly, a CCL5 polymorphism [the CCL5-403 G/A single nucleotide polymorphism (22, 99)] is associated to a higher risk of susceptibility to the onset of the disease, while modified CCL5 ligands are efficacious in controlling the symptoms and the neurodegenerative processes in EAE mice (15).

CCR1, CCR3, and CCR5 exist in different cell types, including T lymphocytes, monocytes/macrophages, and immature dendritic cells, but also exist in neurons and astrocytes (101–104). In particular, CCR1 and CCR3 are expressed by circulating T cells as well as in monocytes, which are occasionally found in perivascular infiltrates in the brain of MS patients. Differently, CCR5-positive T cells and macrophages are concentrated in the active demyelinating lesions in CNS of MS patients (13, 22). To note, the expression of CCRs in CNS correlates with disease severity (105–107) as proved by the observation that clinical symptoms are reduced in CCR1, CCR3, or CCR5 knockout (k.o.) EAE animals, which also suggest redundancy in the chemokine system (7, 8, 99, 108).

GLUTAMATE IN DEMYELINATING DISORDERS

Glutamate is the major excitatory neurotransmitter in CNS, where it mediates important physiological functions (i.e., synaptic plasticity, learning, and memory), but also triggers excitotoxic degenerative processes. Glutamate concentration in the synaptic cleft is finely tuned by several cellular mechanisms including active re-uptake and release from nerve terminals as well as presynaptic mechanisms of control mediated by auto- and/or heteroreceptors (65, 82, 109–116). Glutamate bioavailability is also affected by neighboring astrocytes [i.e., the cells that take up and release the aminoacid (77)] as well as by altered glutamate metabolism. L-Glutamate signaling, however, is not restricted to neuron/astrocyte compartments since glutamate receptors (GluR3-containing AMPA receptors and mGluR1/5 receptors) exist also in immune cells, including cells of the T lineage (117–119). Therefore, besides its role in controlling chemical transmission and excitotoxicity, glutamate may represent a chemo-attractant driving force for the recruitment and the migration of leukocytes and T cells into CNS site where glutamate release occurs (119).

Increased glutamate levels are found in the cerebrospinal fluid of MS patients (119–121) possibly because of the down-regulation of glutamate-metabolizing enzymes (glutamate dehydrogenase and glutamine synthase) and up-regulation of glutamate-producing enzyme glutaminase (122). In 2003, Sarchielli et al. (119) compared the levels of aspartate and glutamate in the CSF of patients suffering from different forms of MS and of controls healthy individuals. The authors find a significant increase of the glutamate levels in patients suffering from the relapsing–remitting form of MS. Interestingly, the glutamate levels were significantly higher in individuals suffering from the relapsing–remitting form of MS with active central lesions during the stable phase than in patients suffering from a similar form of disease, but without lesions. Inasmuch, high levels of glutamate were also detected in patients suffering from the secondary progressive form of MS.

Impaired glutamate bioavailability was also observed in EAE animals. However, depending on the animal model used and the brain region under study, opposite modifications of glutamate release efficiency were observed, consistent with the view that, in demyelinating disorders, impaired glutamate transmission at active synapses is a complex event. Increased glutamate release was detected in the spinal cord of EAE rats (123, 124) as well as in striatal and spinal cord nerve terminals of EAE mice (72, 88, 124–126), while reduced glutamate release was observed in cortical and hippocampal nerve endings of both mice and rats suffering from EAE disease (72, 88, 100, 126, 127). As to glutamate receptors, both metabotropic and ionotropic glutamate receptors (namely mGlu1/5 and mGlu4, mGlu2/3 receptors, and NMDA and AMPA receptors) control glutamate release (83, 109, 113, 115, 116, 128, 129). The expression and the function of these receptors were found to be altered in EAE mice when compared with controls (109, 128–136), suggesting that they represent suitable targets of drugs for the cure of MS symptoms Besides receptors, also glutamate transporter expression is modified in EAE rats (124), determining increased glutamate bioavailability (137) and consequent neurotoxicity.

Generalized ongoing subclinical axonal degeneration in lesioned and non-lesioned white matter, as well as in gray matter, is detectable in CNS of MS patients and seems to occur independently from inflammation or demyelination, representing an

early cause of CNS damage in MS (138). Interestingly, besides the spinal cord, neurodegeneration also takes place in other brain regions, such as the cortex and the hippocampus, and could be responsible of cognitive and affective dysfunctions (121, 139–141) that represent common and early manifestations of MS (142, 143).

CCL5-MEDIATED CONTROL OF GLUTAMATE RELEASE IN EAE MICE

The thesis that central chemokines and classic transmitters functionally cross-talk in CNS has several implications and add new aspects of interest to the role of chemokines in the synaptic derangements that typify neuro-inflammatory central disease.

As already stated, an abnormal overproduction of CCL5 in the spinal cord, and to a lesser extent in the cortex, of EAE mice was evidentiated in immunocytochemistry analysis and confirmed in tissue homogenate (88, 100, 126) and in blood (88). Concomitantly, changes in glutamate exocytosis from nerve endings isolated from the cortex and the spinal cord of EAE mice were observed. Quite interestingly, the modifications of glutamate exocytosis observed in cortical and spinal cord synaptosomes from EAE mice recall the presynaptic modulation elicited by CCL5 in nerve terminals in healthy mice. Actually, glutamate exocytosis was reduced in cortical nerve endings from EAE mice [i.e., in this CNS region, CCL5 inhibits glutamate exocytosis in control animals (66, 71)], but it was drastically increased in spinal cord terminals [where a positive role of CCL5 on glutamate release was observed in healthy animals (66)].

In both CNS regions, the altered glutamate exocytosis was paralleled by impaired second messenger production. Again, the alterations in cAMP ad IP_3 productions observed in both cortical and spinal cord synaptosomal subpopulations from EAE mice were reminiscent of the modulatory presynaptic effects exerted by CCL5 on the corresponding enzymatic pathways in nerve terminals from control, non-immunized, mice. In fact, endogenous cAMP was drastically reduced in cortical synaptosomes but not in the spinal cord, where IP_3 production, the second messenger accounting for the CCL5-mediated presynaptic facilitation of glutamate exocytosis was significantly augmented (**Figure 1**) (66, 88).

The changes in second messenger productions and release efficiency could be explained by assuming that the prolonged *in vivo* CCR activation elicited by the high CCL5 could have triggered adaptive intraterminal changes in nerve terminals (144–146), which are retained in "*ex vivo*, *in vitro*" synaptosomal preparations and can emerge in "*in vitro*" functional studies as changes in glutamate release efficiency and second messenger production. As a matter of fact, the abnormal expression of the chemokine could have reverberated on the CCR repertoire presynaptically located on glutamatergic nerve terminals, leading to adaptation of the CCRs heteromers controlling glutamate exocytosis. These adaptations might lead to changes in the receptor expression and/or associated signaling that might account for the profound changes in glutamate exocytosis in nerve terminals from EAE mice. The CCR composition of the presynaptic chemokinergic oligomers involved in the CCL5-mediated control of glutamate exocytosis in both cortical and spinal cord nerve endings of EAE mice, however, was conserved when compared to control mice, indirectly suggesting that adaptation in CCR subunits assembly were not involved in the EAE-induced changes to the CCL5-mediated control of glutamate exocytosis described above (72).

As to the second messengers, the strict correlation linking CCL5 levels, glutamate release efficiency and IP3, and cAMP accumulation was confirmed by the observation that administration of drugs able to reduce the overexpression of CCL5 in CNS [i.e., the antidepressant desipramine (DMI)] (126) restored both presynaptic functions (i.e., transmitter exocytosis as well as second messenger production) at glutamatergic nerve endings in the cortex of mice suffering from EAE. The beneficial effects exerted by DMI were mediated by the change in noradrenaline bioavailability, due to blockade of the noradrenaline transporters. Actually, the transient increase in the endogenous amine in the synaptic cleft elicited by DMI activates the α and β receptors expressed in astrocytes in the near proximity of the noradrenergic nerve terminals, the activation of which hampers the central endogenous production and release of pro-inflammatory chemokines, including CCL5 (53, 126, 147) from these cells. Interestingly, the peripheral production of the chemokine (which at that stage of disease is already augmented) was unaffected (126).

To conclude, hampering the central overproduction of CCL5 leads to a marked amelioration of the presynaptic defects in terms of release of glutamate and second messenger production. Altogether, these observations clearly support a strict correlation between the increased production of CCL5 in the CNS and the onset of synaptic glutamatergic alteration in EAE mice, also strengthening the pathological role of the "glial to neuron" "CCL5–glutamate" interaction.

CCL5 IN MS: CLINICAL STUDIES

In 2016, Centonze et al. (40) demonstrated that the endogenous concentration of CCL5 in the cerebrospinal fluid of MS patients suffering from the active form of the disease was largely increased when compared to healthy individuals and to patients at the inactive stage of disease. Inasmuch, the authors showed a significant correlation between the endogenous level of CCL5 and the amount of IL-1 beta, used as a marker of gravity of the disease. CSF levels of RANTES were associated with enhanced cortical excitability in the cortex, as suggested by results from studies in which cortical excitability and plasticity was monitored with transcranial magnetic stimulation in MS patients. In these experiments, the authors highlighted a high correlation between the increased intracortical facilitation and the endogenous amount of CCL5. Differently, no correlation emerged when studying the relation linking CCL5 and a long-term potentiation-like synaptic plasticity measured through theta burst stimulation in the same patients. Despite the contrasting observation, the authors concluded that CCL5 couples inflammation and synaptic excitability in MS brains.

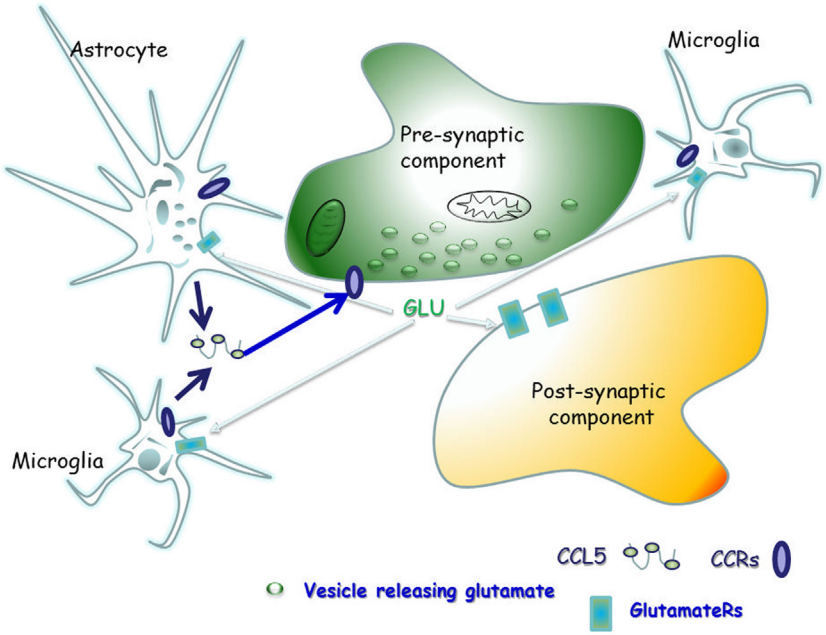

FIGURE 2 | The "quad partite" synapse is a functional structure consisting of neurons, astrocytes, and microglia cells. It represents the simplest central Unit, where adaptive and damaging processes occur in neuro-inflammatory disorders, including the demyelinating one. The concept originates from the tripartite synapse (152), but it is characterized by a highest level of complexity, since microglia is included as players of synaptic derangement. In a simplified model for demyelinating disorder, microglia cells migrating from periphery to central nervous system (CNS) as well as resident central microglia rapidly expand their populations and differentiate into the M1- and M2-cell subgroups, which exert various and mostly opposite functions in the brain. Microglia cells of the M1 group releases pro-inflammatory cytokines, including CCL5, which in turn activate astrocytes. In a whole these events sustain and worsen central inflammatory processes. Differently, M2 microglia secretes anti-inflammatory cytokine and its neuroprotective. Astrocytes are the most abundant glial cells in the human brain and represent the innate immune sentinels that sheath cerebral blood vessels controlling the entry of peripheral cells into the CNS during infection. Astrocytes are neuroprotective at the initial stage of disease, since they reduce local hyper-glutamatergicity by active glutamate uptake processes. Astrocyte activation, however, becomes pathological upon prolonged exposure to the pro-inflammatory compounds released from neighboring microglial cells. At this stage, reactive astrocytes become hypertrophic, do not uptake efficiently glutamate, but release much more cytokines (including CCL5), which accelerate neurodegenerative processes. CCL5 actively released by activated astrocytes and microglia by one side and the abnormal bioavailability of glutamate in the synaptic cleft, on the other side, reverberate onto neurons, eliciting structural and functional changes at chemical synapses.

IMPACT OF DISEASE-MODIFYING DRUGS ON THE ENDOGENOUS PRODUCTION OF CCL5 IN DEMYELINATING DISORDERS

As already stated in this review, CCL5 production is increased in MS patients suffering from both the remitting and the non-remitting form of the disease (15, 41, 89–92). Interestingly, most of the therapeutics currently in use for the cure of MS reduces significantly the overexpression of CCL5.

It is the case of interferon-beta-1b (IFN-beta-1b); administration of this drug prevents CCL5 overproduction in the sera, in the peripheral blood and in the adherent mononuclear cell supernatants during both the relapse and the remission stage of the pathology. These observations are compatible with the idea that CCL5 might be involved in determining the molecular events accounting for the action of IFN-beta-1b in MS patients (38).

Similarly, glatiramer acetate, an approved drug for the treatment of MS, was reported to reduce the TNF-alpha-induced CCL5 mRNA overexpression in human U-251 MG astrocytes. This effect was attributed to the inhibition of mRNA transcription and led to the conclusion that glatiramer acetate may exert its therapeutic effect in MS also by inhibiting pro-inflammatoy signaling (148).

Activation of cannabinoid receptors, which represents a therapeutic approach useful to control the progression of central neuroinflammation in EAE mice and MS patients, also reduces the endogenous availability of the chemokine CCL5 being concomitantly beneficial to the progression of the demyelinating disorder (41, 149).

Laquinimod is a novel orally administered drug for the treatment of relapsing–remitting MS. The molecular events accounting for its therapeutic effects are far from to be elucidated. Monocytes obtained from laquinimod-treated patients tended to secrete lower levels of the pro-inflammatory chemokines CCL2 or CCL5 (150).

Another orally active disease-modifying drug is Fingolimod. Fingolimod is a pro-drug, rapidly metabolized to its active form, the fingolimod-phosphate (fingolimod-P). By acting at the S1PRs in microglia cells, in circulating T cells, and in the spleen, fingolimod-P prevents the egress of lymphocytes and exerts central anti-inflammatory effects favoring remyelination (151). Recent data demonstrated that *in vivo* oral (the drug dissolved in the

drinking water) administration of this drug largely ameliorated the clinical symptoms in EAE mice. The treatment was beneficial to the inflammation and demyelination in the spinal cord of EAE mice, also significantly reducing the endogenous content of CCL5 in this CNS region (100).

Quite interestingly, the abovementioned therapeutics were also described to ameliorate glutamatergic synaptic transmission (152–155) further supporting the strict connection linking CCL5 overexpression and glutamatergic synaptic derangements.

CONCLUSION

The scope of this manuscript is to review the literature concerning the physio-pathological role of CCL5 in controlling glutamate transmission in the CNS of healthy mammals, as well as of individuals and animals suffering from demyelinating disease, in order to highlight the main role of CCL5 as a modulator of the neuroimmune cross-talk in the "quad partite" synapse in CNS (**Figure 2**).

These effects, together with the well-known chemo-attractant role of the chemokine toward glial cells, suggest that CCL5 exerts a dual role in the CNS of individuals suffering from MS. On one hand, the chemokine impairs the chemical transmission at asymmetric synapses in selected region of the CNS. On the other hand, it worsens the course of disease progression by favoring the recruitment of pro-inflammatory glial cells in the site of the lesion.

When considering its role as modulator of glutamate transmission, the chemokine preferentially emerges as a key effector of the "astrocytes to neurons" signaling in CNS. Actually, the chemokine released from astrocyte and microglia is an efficient paracrine modulator of glutamate release at synaptic boutons of glutamatergic neurons in both healthy and demyelinating conditions, while its autocrine role of modulator of glutamate overflow from astrocytes is less evidnet (**Table 1**).

Therapeutic approaches aimed at containing the overexpression of the chemokine might represent therefore a useful approach to the cure of MS.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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