CLINICAL RELEVANCE OF THE IMMUNE-TO-BRAIN AND BRAIN-TO-IMMUNE COMMUNICATIONS

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CLINICAL RELEVANCE OF THE IMMUNE-TO-BRAIN AND BRAIN-TO-IMMUNE COMMUNICATIONS

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Experimental and clinical evidence demonstrates an intense crosstalk among the nervous, endocrine and immune systems. The central nervous system (CNS) not only has the capacity to affect peripheral immune function, but is also able to sense and process signals from the peripheral immune system. The bi-directional interaction between the CNS and the peripheral immune system has gained great interest as it can help better understand disease pathophysiology as well as improving health and treatment outcomes in patients. On the one hand, inflammatory factors are known to affect CNS functions and to induce neuropsychiatric symptoms, making immune-to-brain communication highly relevant for psychiatric diseases and their treatments. On the other hand, analyzing pathways of brain-to-immune communication will help to understand the pathophysiology of chronic inflammatory disorders and will form the basis for optimizing treatment of these diseases.

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CLINICAL RELEVANCE OF BRAIN-TO-IMMUNE COMMUNICATIONS

frontiers in Behavioral Neuroscience

EDITORIAL
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Editorial: Clinical Relevance of the Immune-to-Brain and Brain-to-Immune Communications

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Keywords: immune system, brain, brain-immune interactions, immunopsychiatry, stress, psychoneuroimmunology, gut-brain axis

Editorial on the Research Topic

Clinical Relevance of the Immune-to-Brain and Brain-to-Immune Communications

Experimental and clinical evidence demonstrates an intense crosstalk among the nervous, endocrine and immune systems (Dantzer, 2018). The central nervous system (CNS) not only has the capacity to affect peripheral immune functions, but is also able to sense and process signals from the peripheral immune system. The bi-directional interaction between the CNS and the peripheral immune system has gained great interest as it can help better understanding disease pathophysiology to improve health and treatment outcomes in patients, and to understand how modifiable life-style factors can be associated with health. In this special issue of *Frontiers in Behavioral Neuroscience, Frontiers in Immunology,* and *Frontiers in Neurology,* we have collected original works and perspectives that provide new insights on the clinical relevance of immune-to-brain and brain-to-immune communications.

CLINICAL RELEVANCE OF IMMUNE-TO-BRAIN COMMUNICATIONS

In order to provide improved understanding of possible underlying mechanisms of inflammation-associated depression (Lotrich, 2015), Kotulla et al. studied specific symptoms of negative self-schema (general self-esteem, intolerance of uncertainty, and hopelessness) in response to an inflammatory challenge. Although inflammation lowered mood, negative self-schema was not significantly impacted. This indicates that negative self-schemata might not be a mechanism by which inflammation leads to depression. In their review, Lacourt et al. provide new insights on the mechanisms underlying chronic fatigue. They propose that the chronic activation of inflammatory processes at a low-grade state, such as observed in chronic fatigue syndrome (Fletcher et al., 2009) and cancer survivors (Bower, 2007), might contribute to persistent fatigue via two main mechanisms: changes in metabolism, leading to reduced glucose availability and thus to reduced cellular energy; and increased behavioral energy expenditure. Hence, an imbalance between energy expenditure and available resource could lead to persistent fatigue. Koenig et al. investigated the increased brain vulnerability to psychosocial stress during aging, which is a risk factor for the development of mental illness (De Kloet et al., 2005). Using young and old rats subjected to psychological novel environment stress and to lipopolysaccharide administration, the authors showed dysregulated pituitary cytokine interactions and brain cell activation in the hypothalamus-pituitary-adrenal axis in old rats.

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This mechanism could underlie the prolonged and maladaptive response to stressors that is observed in aging.

As highlighted by Tong et al. and Oberstein et al., peripheral immune processes might also be involved in CNS diseases. These authors assessed the peripheral immune profile of two respective patient populations. Tong et al. found that patients with Neuromyelitis Optica Spectrum Disorders (NMOSD), a demyelinating disease affecting optic nerve and spinal cord, exhibited higher plasma levels of pro-inflammatory cytokines and of eosinophil chemoattractants, which was, in turn, associated with the number of relapses. The authors suggest that inflammatory activity could contribute to the pathophysiology of NMOSD by increasing eosinophil chemoattractants, and thus favoring eosinophil infiltration, which is a specific feature of NMOSD (Zhang and Verkman, 2013). Oberstein et al. report an increased proportion of Th17 cells in the blood, as well as a significant correlation between blood proportion of regulatory T cells and the levels of Tau in the cerebrospinal fluid, in patients with mild cognitive impairment due to Alzheimer's Disease (AD), but not in patients with mild cognitive impairment with negative CSF AD's biomarkers and in healthy controls. This indicates a potential role of the adaptive immune system in the pathophysiology of AD.

The studies from Herz et al. and Tadros et al. reveal the importance of the immune system for the central nervous system during the neonatal period. Based on previous research (e.g., Nazmi et al., 2018), Herz et al. hypothesized that the depletion of peripheral T cells would protect against consequences of hypoxic-ischemic injury in the neonatal brain of mice. However, contrary to the hypothesis, the depletion of peripheral T cells led to an exacerbation of the effects of hypoxic-ischemic brain injury in mice neonates, with an increased loss of gray and white matter, along with an increased brain infiltration of innate immune cells. Thus, the observed infiltration of T cells after hypoxic-ischemic injury could actually be protective in neonates. Tadros et al. assessed whether changes in the properties of superficial dorsal horn (SDH) neurons is a mechanism by which an immune challenge during the neonatal period leads to heighten inflammatory pain later in life. The authors found that frequency of spontaneous excitatory synaptic currents of SDH neurons was increased in the group of rats having received the neonatal immune challenge compared to saline. This reflects a hyper-excitability of the SDH neuronal network, which could contribute to the altered nociceptive signaling observed after a neonatal immune challenge.

Gut immunity and the gut-brain axis play an important role in modulating CNS functions (Grenham et al., 2011). In their review, Bajic et al. describe how the gut-brain axis is likely to be involved in the psychological and neurological complications of chemotherapy. Chemotherapy induces mucositis, associated with an inflammatory response, changes in the gut microbiome, increased intestinal permeability, and damages to the nerves of the myenteric plexus. These changes cause a disruption in the gut-brain axis, which is known to alter mood and cognition. Langgartner et al. show that rectal transplantation of feces from non-stressed mice partially reversed the physiological (thymus atrophy, low-grade inflammation, altered bone homeostasis) and behavioral (anxiety) effects of chronic psychosocial stress (induced by housing with a dominant CD-1 mouse). This indicates a potential role of gut microbiota in some of the physiological and behavioral changes induced by chronic psychosocial stress.

CLINICAL RELEVANCE OF BRAIN-TO-IMMUNE COMMUNICATIONS

Dysregulation of peripheral innate immune responses is observed in stress-associated psychiatric disorders, such as major depression (Dowlati et al., 2010). Ambrée et al. studied whether vulnerability to stress was associated with specific differences in the innate immune system compared to stress resilience. In mice chronically exposed to social defeat, the authors showed that the innate peripheral and brain immune cells of vulnerable mice (i.e., those that show altered social behavior after social defeat) presented a specific inflammatory profile that was not observed in resilient mice. These findings show a strong interaction between stress vulnerability and immunological consequences.

The effect of psychosocial stress on immune functions is studied to a large extent in rodents. In their review, Gimsa et al. propose instead to use pigs as an animal model in this field. Many anatomical and physiological characteristics, such as brain anatomy (Lind et al., 2007), stress hormone (cortisol), and immune functions (Meurens et al., 2012), are more similar to humans in pig models than rodents. To support their approach, the authors present data on short-term and long-term effects of social stress in preclinical pig models regarding immunity and neuroendocrine regulation, as well as consequences for health and well-being.

Macrophages are central immune cells contributing to innate and adaptive immunity. In a comprehensive review, Jurberg et al. thoroughly describe how neurotransmitters and neuroendocrine hormones influence macrophage physiology and functioning, such as inflammatory responses, macrophage maturation and polarization, and migration. The authors also discuss how these mechanisms could be targeted in the development of therapies for inflammatory diseases and cancer.

Lastly, Willekens et al. build upon the known brain-to-immune communication networks to propose mechanisms by which mindfulness-based interventions might improve multiple sclerosis (MS) outcomes. Mindfulness has been shown to improve quality of life, depression and fatigue in MS patients, although the underlying mechanisms remain unknown. The authors provide data supporting immunomodulatory effects of this treatment, and call for the measurement of immunological and neuroimaging biomarkers in studies assessing the effect of mindfulness in MS.

CONCLUSION

The articles combined in this special issue highlight the central role of immune-to-brain and brain-to-immune communications in various clinical pathologies, including, but not limited to, depression, anxiety, fatigue, pain, stress-related disorders,

neurological diseases, and inflammatory diseases. This special issue also reveals the importance of clinical *and* animal studies, along with review articles reflecting on the current knowledge, in the quest for a better comprehension of the brain-immune mechanisms and their clinical relevance.

AUTHOR CONTRIBUTIONS

JL and MH have written the draft of this editorial. MS and ML have provided critical feedback. All authors approve the final version.

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ORIGINAL RESEARCH
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Does Human Experimental Endotoxemia Impact Negative Cognitions Related to the Self?

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A role of inflammatory processes in the pathophysiology of depression is increasingly recognized. Experimental endotoxemia offers an established model to induce transient systemic inflammation in healthy humans, and has been proposed as an experimental paradigm of depression. Indeed, different symptoms of depression can be observed during experimental endotoxemia, including negative mood or dysthymia as key symptoms of depression. Hopelessness and low self-esteem constitute common cognitive symptoms in depression, but have not been specifically assessed during endotoxemia. Thus, we pooled data from healthy volunteers who received low-dose endotoxin (i.e., 0.4 or 0.8 ng/kg lipopolysaccharide, LPS) or placebo in three randomized, controlled studies to investigate the effects of LPS on cognitive schemata related to depression. Validated questionnaires were used to assess self-esteem, hopelessness and the vulnerability factor intolerance of uncertainty after intravenous injection of LPS or placebo. Plasma tumor necrosis factor (TNF)-α and interleukin (IL)-6 were repeatedly assessed, along with self-reported mood. Because not all questionnaires were available from primary studies, data were analyzed in two separate data sets: In data set 1, self-esteem and intolerance of uncertainty were assessed in N = 87 healthy volunteers, who randomly received either 0.4 or 0.8 ng/kg LPS or placebo. In data set 2, hopelessness was measured in N = 59 volunteers who randomly received either LPS (0.8 ng/kg) or placebo. In both data sets, LPS-application led to significant increases in TNF-α and IL-6, reflecting systemic inflammation. Positive mood was significantly decreased in response to LPS, in line with inflammation-induced mood impairment. General self-esteem, intolerance of uncertainty and hopelessness did not differ between LPS- and placebo groups, suggesting that these negative cognitive schemata are not responsive to acute LPS-induced systemic inflammation. Interestingly, LPS-treated volunteers reported significantly lower body-related self-esteem, which was associated with increased TNF-α concentration. Thus, certain aspects of self-esteem related to physical attractiveness and sportiness were reduced. It is conceivable that this effect is primarily related to physical sickness symptoms and reduced physical ability during experimental endotoxemia. With respect to cognitive symptoms

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of depression, it is conceivable that LPS affects cognitive *processes*, but not negative cognitive schemata, which are rather based on learning and repeated experiences.

Keywords: systemic inflammation, lipopolysaccharide, cytokines, TNF-α, depression, mood, hopelessness, self-esteem

INTRODUCTION

Major depression (MD) is a prevalent and severe psychiatric disorder characterized by depressed mood, loss of interest or pleasure, changes in weight, appetite, sleep and activity, fatigue and suicidality (Kessler et al., 2005; American Psychiatric Association, 2013; Hasin et al., 2018). Another key feature of depression is neurocognitive symptoms. These not only include changes in information processing, impaired concentration and indecisiveness, but also negative cognitions related to the self, the past, present and future, contributing to feelings of worthlessness, hopelessness and excessive or inappropriate guilt (Clark and Beck, 2010; American Psychiatric Association, 2013; Uher et al., 2014), and ultimately to depression onset and maintenance (Evans et al., 2005; Disner et al., 2011). The pathogenesis of MD remains incompletely understood, which hampers the development of new therapeutic approaches (DellaGioia and Hannestad, 2010).

A role of inflammatory processes in MD pathophysiology is increasingly recognized (Capuron and Miller, 2004; DellaGioia and Hannestad, 2010; Kiecolt-Glaser et al., 2015; Yirmiya et al., 2015; Miller and Raison, 2016; Otte et al., 2016), offering treatment perspectives for at least a subgroup of patients. Anti-inflammatory treatment reportedly improves symptoms in patients resistant to classical anti-depressant medication (Tyring et al., 2006; Miller and Raison, 2016). Elevated inflammatory markers, such as C-reactive protein (CRP) and the pro-inflammatory cytokine interleukin (IL)-6 have been demonstrated in a substantial proportion of MD patients (Howren et al., 2009; DellaGioia and Hannestad, 2010; Dowlati et al., 2010). In depression-free individuals, increased concentrations of inflammatory markers can independently predict the incidence of depression over a time period of 10 or more years (Gimeno et al., 2009; Pasco et al., 2010; Raison and Miller, 2011). Finally, MD is more prevalent in patients with chronic inflammation (Patten et al., 2003; Graff et al., 2009; Matcham et al., 2013). It remains however unclear if inflammatory processes are related to all symptom domains of MD or may rather be associated with only specific symptoms, calling for preclinical research.

The experimental administration of low-dose endotoxin (e.g., lipopolysaccharide, LPS) constitutes an established translational model to analyze the effects of systemic inflammation on mood, cognition and behavior (Andreasen et al., 2008; Schedlowski et al., 2014; Suffredini and Noveck, 2014; Lasselin et al., 2018). LPS is a cell-wall component of Gram-negative bacteria that activates the innate immune system through a Toll-like receptor 4-dependent pathway, ultimately leading to a systemic increase in pro-inflammatory cytokines (Andreasen et al., 2008; Schedlowski et al., 2014; Zouikr and Karshikoff, 2017). Via vagal and humoral afferent pathways, these cytokines act on the brain and induce changes in mood and behavior, which are commonly referred to as sickness behavior (Dantzer and Kelley, 2007; Schedlowski et al., 2014). Sickness behavior shows striking similarities to several key symptoms of depression, although sickness behavior symptoms are transient and typically disappear when inflammation has resolved (Dantzer et al., 2008; Raison and Miller, 2011). Low doses of endotoxin reliably induce transient symptoms including negative mood (Reichenberg et al., 2001; Wright et al., 2005; Eisenberger et al., 2009, 2010b; Hannestad et al., 2011; Benson et al., 2017a; Engler et al., 2017), feelings of social isolation and disturbed psychosocial functioning (Eisenberger et al., 2009, 2010b; Inagaki et al., 2015; Moieni et al., 2015a,b), changes in motivation (Eisenberger et al., 2010a; Lasselin et al., 2017) and fatigue (Benson et al., 2017b).

Whether experimental endotoxemia also impacts neurocognitive processes relevant to the characteristic negative cognitive schemata of worthlessness, low self-esteem, guilt and hopelessness in MD remains unclear. It has been proposed that these symptoms may not change in response to LPS (DellaGioia and Hannestad, 2010), however first findings (Eisenberger et al., 2009) showed LPS-induced changes in the Profile of Mood States (POMS) depression scale, a subscale which comprises items related to worthlessness and hopelessness. Further, we recently documented a negative cognitive bias, i.e., a prolonged and more sustained processing of negative information during low-dose endotoxemia (Benson et al., 2017a). This effect was only seen when sad mood was experimentally induced during systemic inflammation using a mood induction paradigm (Benson et al., 2017a). Together, these findings support the notion that systemic inflammation not only increases the susceptibility to negative emotional stimuli, but also changes the cognitive processing of negative information (Bollen et al., 2017). However, this is indirect evidence, and no dedicated data exist thus far in the experimental human endotoxemia literature to address negative cognitive schemata. We herein aim to close this research gap by exploring effects of LPS on self-esteem, hopelessness, and intolerance of uncertainty. Intolerance of uncertainty reflects the tendency to consider possible negative events as frightening or burdening, and constitutes a vulnerability factor for depression and anxiety disorders (McEvoy and Mahoney, 2011; Carleton et al., 2012; Boelen and Lenferink, 2018; Lauriola et al., 2018). Symptoms were assessed with validated questionnaires in a comparatively large cohort of healthy volunteers who participated in double-blind, placebo-controlled LPS studies.

MATERIALS AND METHODS

Participants

Data from a total of N = 109 healthy volunteers (89 men, 20 women) who were randomized to receive either low-dose LPS (0.4 or 0.8 ng/kg body weight) or saline (placebo) were analyzed. All volunteers participated in one of three randomized, double-blind endotoxemia studies (Wegner et al., 2015; Benson et al., 2017a, unpublished data), and completed validated questionnaires to analyze negative cognitive schemata related to depression, along with changes in mood. For cross-over studies (Wegner et al., 2015; Benson et al., 2017a), only data from the first study day was included to avoid carry-over effects (i.e., data from the second day were discarded for the purposes of this analysis). Identical methods were used to measure cytokine concentrations and mood, which allows the pooling of data across studies. Questionnaire data on self-esteem and intolerance of uncertainty were available from two studies with a total of N = 87 participants (Wegner et al., 2015; Benson et al., unpublished data), and were pooled for analysis to *data set 1*. Hopelessness was measured in N = 59 volunteers (Benson et al., 2017a unpublished data), and data were pooled to *data set 2*. All studies were conducted in accordance with the Declaration of Helsinki and were approved by the Institutional Ethics Review Board of the Medical Faculty of the University of Duisburg-Essen (permit numbers: 09-4271; 15-6234-BO; 15-6533-BO). All participants provided written informed consent and received financial compensation for study participation.

Recruitment and Safety Routine

For all primary studies, recruitment, inclusion and exclusion criteria and safety routine were identical and have previously been reported in detail (Wegner et al., 2015; Benson et al., 2017a). Briefly, healthy volunteers aged 18–45 years were recruited via public announcements and underwent an in-depth screening procedure consisting of a structured telephone interview, a physical examination and personal interview conducted by a physician, and repeated laboratory assessments (blood cell count, liver enzymes, renal parameters, electrolytes, coagulation factors and CRP). General exclusion criteria were any pre-existing or current physical or psychiatric illness, pregnancy, body mass index (BMI) <18 or ≥ 29 kg/m^2, current medications, smoking or regular alcohol use (>4 drinks per week). Female participants were only included when taking oral contraceptives to prevent confounding effects of menstrual cycle phase. Pregnancy was ruled out with a urinary pregnancy test on the study day. A diagnosis of depression or clinically-relevant depression symptoms exceeding published cut-off scores of the Beck Depression Inventory (BDI; Hautzinger et al., 1995) were exclusionary for ethical reasons, and also to avoid confounding effects on primary outcome variables, i.e., cognitive symptoms of depression. Participants were told to refrain from strenuous exercise 48 h prior to study days. Safety measures included monitoring for at least 6 h and follow-up examinations 24 h and 7 days after the injection of LPS or placebo.

Study Protocol

Data set 1 consisted of N = 87 healthy male and female volunteers who were randomized to receive either 0.4 ng/kg (N = 29) or 0.8 (N = 20) ng/kg body weight LPS or placebo (N = 38). *Data set 1* includes data from N = 20 female volunteers who received either 0.4 ng/kg body weight LPS (n = 10) or placebo (n = 10). *Data set 2* comprised data from N = 59 healthy men who received either 0.8 ng/kg body weight LPS (N = 25) or placebo (N = 34). LPS (reference standard endotoxin from *Escherichia coli*, serotype O113:H10:K-negative, lot H0K354, United States Pharmacopeia, Rockville, MD, USA; LPS conditions) or saline (placebo condition) was injected via an intravenous catheter placed in an antecubital forearm vein. Blood samples for cytokine analyses were collected before (baseline, BL) as well as 1, 2, 3, and 6 h after injection of LPS or placebo. Body temperature (with an intra-aural thermometer), blood pressure, and heart rate were assessed after blood sampling. Changes in self-reported mood (positive vs. negative) were assessed with the respective subscale of the standardized and validated German multidimensional mood questionnaire (MDBF; Steyer et al., 1997) before (BL) as well as 3 and 6 h after injection of LPS or placebo. Cognitive symptoms of depression were assessed with three validated questionnaires (see below) 3 h post injection. This time point was chosen for two reasons: First, increased plasma concentrations of tumor necrosis factor (TNF)-α and IL-6 were consistently observed 2–4 h post injection in previous studies, indicating systemic immune activation (Wegner et al., 2014; Benson et al., 2017b). More importantly, we found a significant rise in IL-6 in the cerebrospinal fluid 3 h post-injection (Engler et al., 2017), reflecting a CNS response to peripheral inflammation.

Cognitive Symptoms of Depression

Self-Esteem/Self-Worth (Data Set 1)

Self-esteem was assessed with the Multidimensional Self-Worth Scale (MSWS; Schütz et al., 2006), a German adaption of the Multidimensional Self-Concept Scale (MSCS; Fleming and Courtney, 1984). The MSWS contains 32 seven-point Likert-scaled items. The MSWS consists of six subscales, which can be combined to a "general self-esteem" (AWS) scale and a "body-related self-esteem" (KSW) scale. General self-esteem comprises aspects of "emotional," "social," "conflict-related," and "performance-related self-esteem," and body-related self-esteem refers to "self-regarded physical attractiveness" and "self-regarded sportiness." The reliability for the "general self-esteem" (AWS) scale (Cronbach's α = 0.92) and "body-related self-esteem" (KSW) scale (Cronbach's α = 0.85) can be consider as good based on internal reliability (Cronbach's α, Schütz et al., 2006).

Intolerance of Uncertainty (Data Set 1)

Intolerance of uncertainty reflects the tendency to consider possible negative events as frightening or burdening, and constitutes a vulnerability factor for depression and anxiety disorders (McEvoy and Mahoney, 2011; Carleton et al., 2012; Boelen and Lenferink, 2018; Lauriola et al., 2018). Intolerance of uncertainty was assessed with the validated German

UI-18 ("*Unsicherheitsinteroleranz-Skala*," Gerlach et al., 2008) questionnaire, an adaption of the Intolerance of Uncertainty Scale (Freeston et al., 1994). The UI-18 contains 18 items which are answered on a 5-point-Likert-scale, and can be combined to the three subscales "reduced ability to act due to intolerance of uncertainty," "burden due to intolerance of uncertainty" and "vigilance due to intolerance of uncertainty" (Gerlach et al., 2008). All scales assess how people react on uncertainties of life. The scales "burden" and "vigilance" were shown to reliably predict worrying (Gerlach et al., 2008). Reliability for subscales (Cronbach's $\alpha \geq 0.80$), and the overall internal reliability (Cronbach's $\alpha = 0.90$) can be considered as good (Gerlach et al., 2008).

Hopelessness (Data Set 2)

Hopelessness was measured with the revised version of the validated German Hopelessness-scales (H-R-scale; *Skalen zur Erfassung von Hoffnungslosigkeit*; Krampen, 2004). The H-R-scale was theoretically based on Beck ś cognitive theory of depression (Beck et al., 1979). The scale assesses the tendency to evaluate the future in negative terms, and therefore reflects a pessimistic cognitive style. The H-R-scale contains 20 six-point Likert-scaled items. Sufficient to good internal consistency has been reported (Cronbachs $\alpha = 0.74$—$\alpha = 0.92$) for different reference samples).

Plasma Cytokine Concentrations, Leukocyte Counts and C-Reactive Protein

Blood for cytokine analyses was collected in EDTA-treated tubes (S-Monovette, Sarstedt, Nümbrecht, Germany). Plasma was immediately separated by centrifugation (2,000 *g*, 10 min, 4°C) and stored at −80°C until analysis. Plasma TNF-α and IL-6 concentrations were measured using enzyme-linked immunosorbent assays (ELISA; Human Quantikine ELISA, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. The sensitivity of the assays was 0.11 pg/ml for TNF-α and 0.70 pg/ml for IL-6. Mean inter- and intra-assay coefficients of variation were ≤10%. In data set 1, one TNF-α and three IL-6 samples were below the respective detection limits, as well as in data set 2 two TNF-α samples and one IL-6 sample. White blood cell (WBC) counts were determined using an automated cell counter (Sysmex KX-21N, Norderstedt, Germany). CRP concentration was measured before and 24 h post-injection with a polyethylene glycol (PEG)-enhanced immunoturbidimetric assay (sensitivity 0.5 mg/dl) by the Division of Laboratory Research of the University Hospital Essen (Germany).

Statistical Analyses

Data analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and the level of significance was set at $\alpha < 0.05$. Normal distribution was tested using Kolmogorov-Smirnov-test, and non-normally distributed variables (i.e., plasma cytokines) were log-transformed before analysis. Data are shown as mean ± standard error of the mean (SEM). BL sociodemographic and psychological parameters of LPS and placebo groups were compared with univariate analysis of variance (ANOVA; data set 1) or unpaired *t*-tests (data set 2). LPS effects on plasma cytokines, body temperature and mood were analyzed in both data sets with repeated measures ANOVA (rm-ANOVA) with endotoxin condition as group (LPS vs. placebo) and time as within-subject factor. Greenhouse-Geisser correction was applied where appropriate. Bonferroni-corrected *post hoc* unpaired *t*-tests (two-tailed) were computed in case of significant rm-ANOVA interaction effects. To assess group differences in cognitive symptoms of depression (i.e., self-esteem, intolerance of uncertainty, hopelessness) analyses of covariance (ANCOVA) were computed. To exclude that effects were confounded by inter-individual differences in BL depressive symptoms, BL BDI scores were entered as a covariate in these analyses. As an indicator of effect sizes, partial Eta^2 (η_p^2) was computed for ANOVA/ANCOVA. Partial Eta^2 allows to estimate effect sizes within the relevant population as it indicates the percent of variance of measures (e.g., self-esteem scores), which is explained by a respective factor (e.g., LPS vs. placebo; Fields, 2013). Correlations were computed as Pearson's *r*.

RESULTS

Sample Characteristics

Data set 1 comprised $N = 87$ healthy volunteers (20 women) with a mean age of 26.9 ± 0.1 years and a mean BMI of 23.6 ± 0.3 kg/m^2. *Data set 2* consisted of $N = 59$ healthy men with a mean age of 26.5 ± 0.6 years and mean BMI of 24.1 ± 0.3 kg/m^2. No differences in age, BMI or BL BDI scores were observed between LPS- and placebo groups (**Table 1**).

Plasma Cytokines and Body Temperature

In both data sets, LPS administration expectedly led to transient increases in plasma cytokine and CRP concentrations, leukocyte counts and body temperature (see **Figure 1** for data set 1 and **Figure 2** for data set 2). Specifically, LPS application induced a significant increase in plasma concentrations of TNF-α (*data set 1*: $F = 29.4$, $p < 0.001$, $\eta_p^2 = 0.41$; *data set 2*: $F = 60.0$, $p < 0.001$, $\eta_p^2 = 0.62$) and IL-6 (*data set 1*: $F = 47.7$, $p < 0.001$, $\eta_p^2 = 0.54$; *data set 2*: $F = 80.5$, $p < 0.001$, $\eta_p^2 = 0.59$), along with a significant rise in circulating leukocyte numbers (*data set 1*: $F = 29.4$, $p < 0.001$, $\eta_p^2 = 0.42$; *data set 2*: $F = 44.5$, $p < 0.001$, $\eta_p^2 = 0.44$), body temperature (*data set 1*: $F = 14.1$, $p < 0.001$, $\eta_p^2 = 0.25$; *data set 2*: $F = 10.9$, $p < 0.001$, $\eta_p^2 = 0.16$), and CRP concentrations (*data set 1*: $F = 122.1$, $p < 0.001$, $\eta_p^2 = 0.75$; *data set 2*: $F = 121.8$, $p < 0.001$, $\eta_p^2 = 0.69$; all ANOVA interaction effects). *Post hoc* Bonferroni-tests revealed significant differences between LPS groups and placebo groups in both data sets, but not between 0.4 and 0.8 ng/kg LPS groups (for results of *post hoc* tests, see **Figures 1**, **2**).

LPS Effects on Mood

LPS administration led to a transient decline in positive mood in response to LPS (*data set 1*: $F = 8.4$, $p < 0.001$,

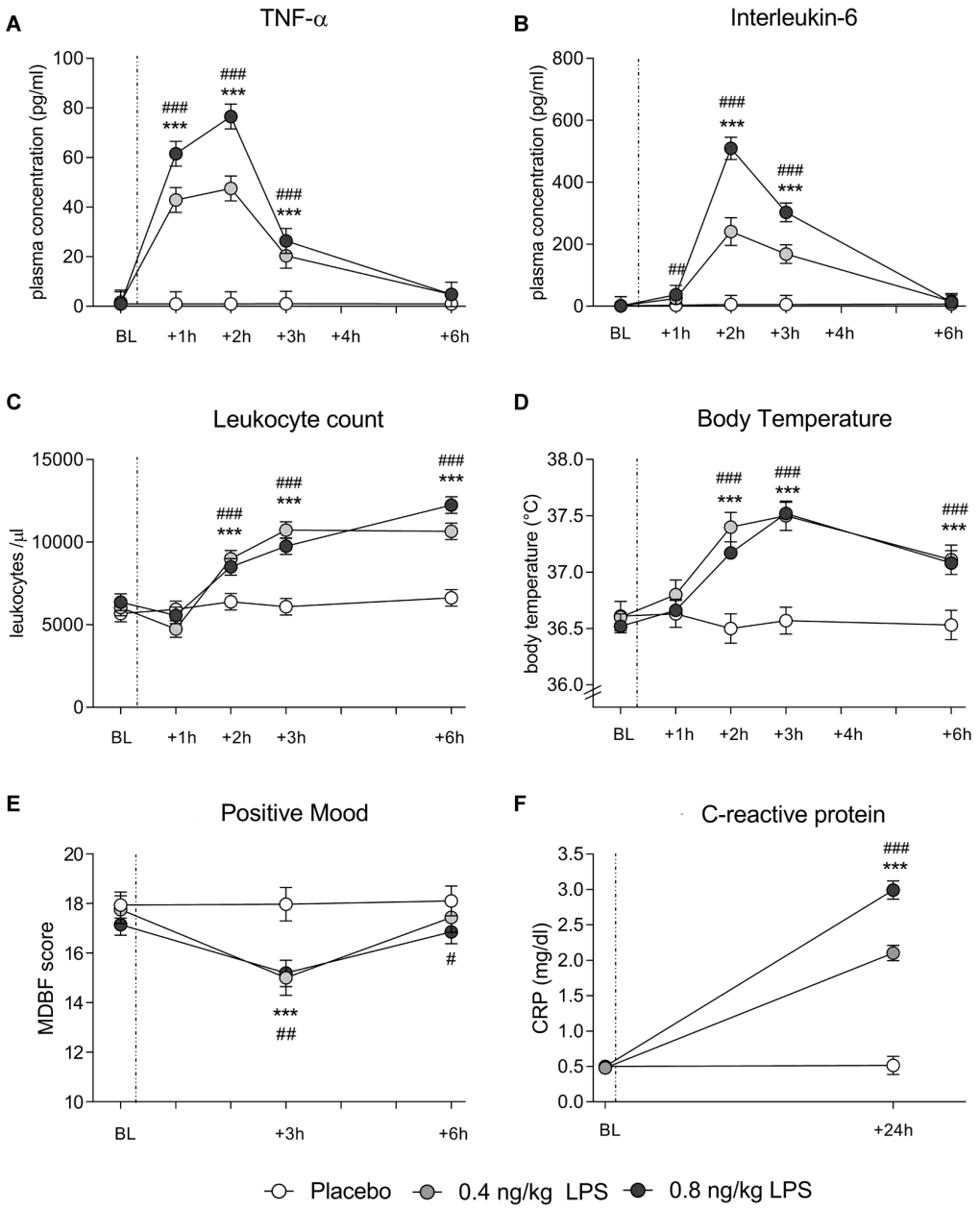

FIGURE 1 | Data set 1: plasma concentrations of tumor-necrosis-factor (TNF)-α **(A)** and Interleukin (IL)-6 **(B)** leukocyte counts **(C)** and body temperature **(D)** were measured before (baseline, BL), and 1, 2, 3 and 6 h after injection of either 0.8 ng/kg body weight lipopolysaccharide (LPS; black dots), 0.4 ng/kg body weight LPS (gray dots), or saline (placebo group; white dots). Mood **(E)** was assessed with the respective subscale of the German Multi-Dimensional Mood (MDBF) questionnaire at BL, as well as 3 and 6 h after injection. C-reactive protein (CRP) **(F)** was measured at BL and 24 h post injection. $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ 0.8 ng/kg LPS group vs. placebo group. $^{***}p < 0.001$, 0.4 ng/kg LPS group vs. placebo group. For results of repeated measures analysis of variance (ANOVA), see text.

$\eta_p^2 = 0.16$; *data set 2*: $F = 10.7$, $p < 0.001$, $\eta_p^2 = 0.15$; ANOVA interaction effects). *Post hoc* Bonferroni tests supported that mood was significantly impaired in the LPS groups when compared to the placebo groups in both data sets. No differences were found between 0.4 and 0.8 ng/kg LPS groups in data set 1 (for results of *post hoc* tests, see **Figures 1**, **2**).

LPS Effects on Cognitive Symptoms of Depression

Self-Esteem (Data Set 1)

General (**Figure 3A**) and body-related (**Figure 3B**) self-esteem were assessed with the validated MSWS questionnaire. Participants who received LPS reported a significantly lower

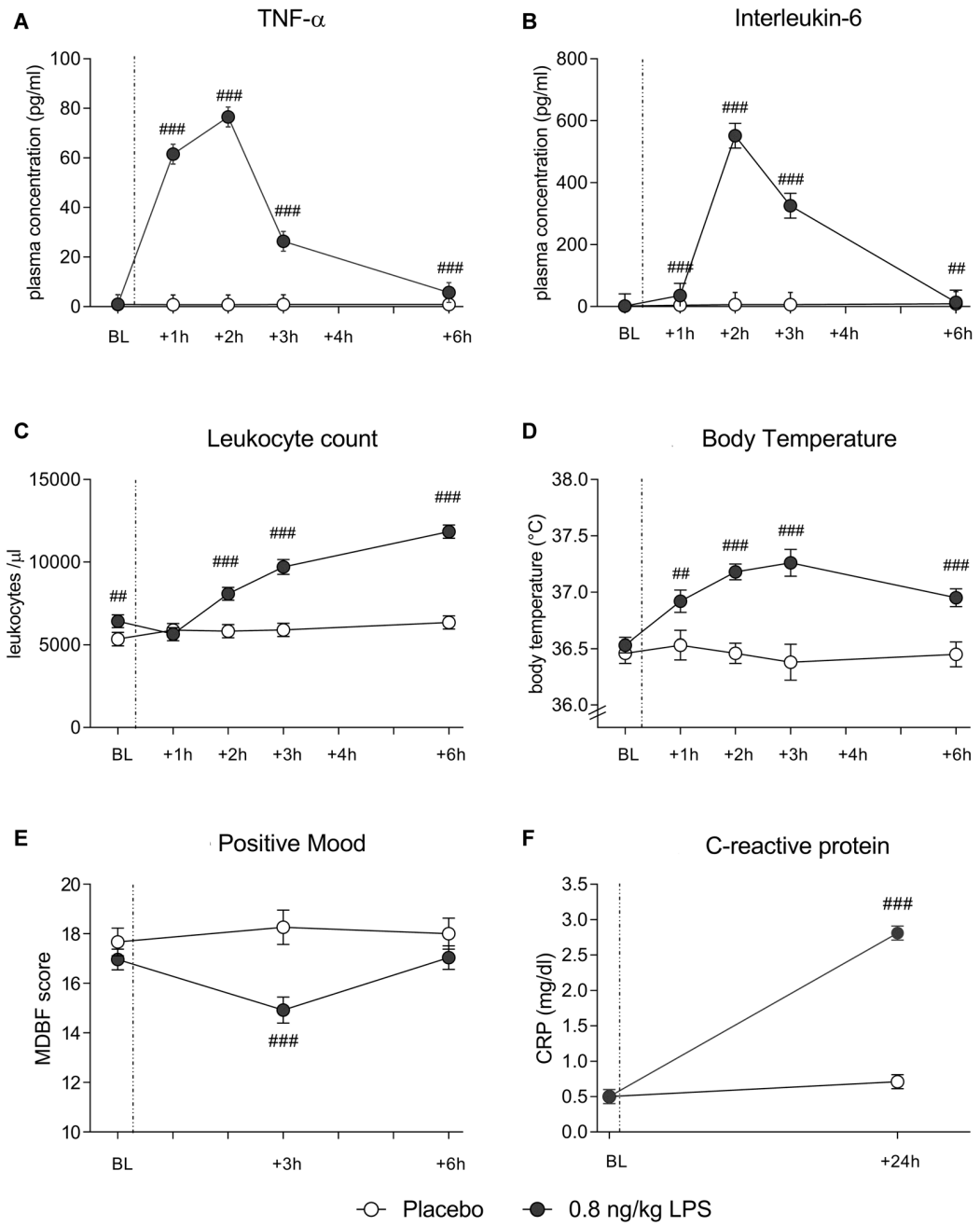

FIGURE 2 | Data set 2: plasma concentrations of TNF-α **(A)** and IL-6 **(B)**, leukocyte counts **(C)** and body temperature **(D)** were measured before (BL), and 1, 2, 3 and 6 h after injection of either 0.8 ng/kg body weight LPS (black dots) or saline (placebo group; white dots). Mood **(E)** was assessed with the respective subscale of the German Multi-Dimensional Mood (MDBF) questionnaire at BL, as well as 3 and 6 h after injection. CRP **(F)** was measured at BL and 24 h post injection. $^{###}p < 0.001$, $^{##}p < 0.01$, 0.8 ng/kg LPS group vs. placebo group. For results of repeated measures ANOVA, see text.

body-related self-esteem ($F = 3.57$, $p = 0.03$, $\eta_p^2 = 0.08$). *Post hoc* testing revealed that the higher LPS group (0.8 ng/kg) displayed significantly lower scores for body-related self-esteem compared to the placebo group ($p = 0.03$), while the lower dose LPS group (0.4 ng/kg) differed neither from the placebo ($p = 0.60$) nor from the 0.8 ng/kg LPS dose group ($p = 0.49$). LPS and placebo groups did not differ in general self-esteem ($F = 0.55$, $p = 0.57$, $\eta_p^2 = 0.01$). Exploratory correlation analysis within LPS-treated volunteers revealed a significant association between higher TNF-α concentrations 3 h post injection and lower body-related self-esteem (MSWS-scores; $r = -0.31$, $p < 0.05$, **Figure 3C**). No further significant

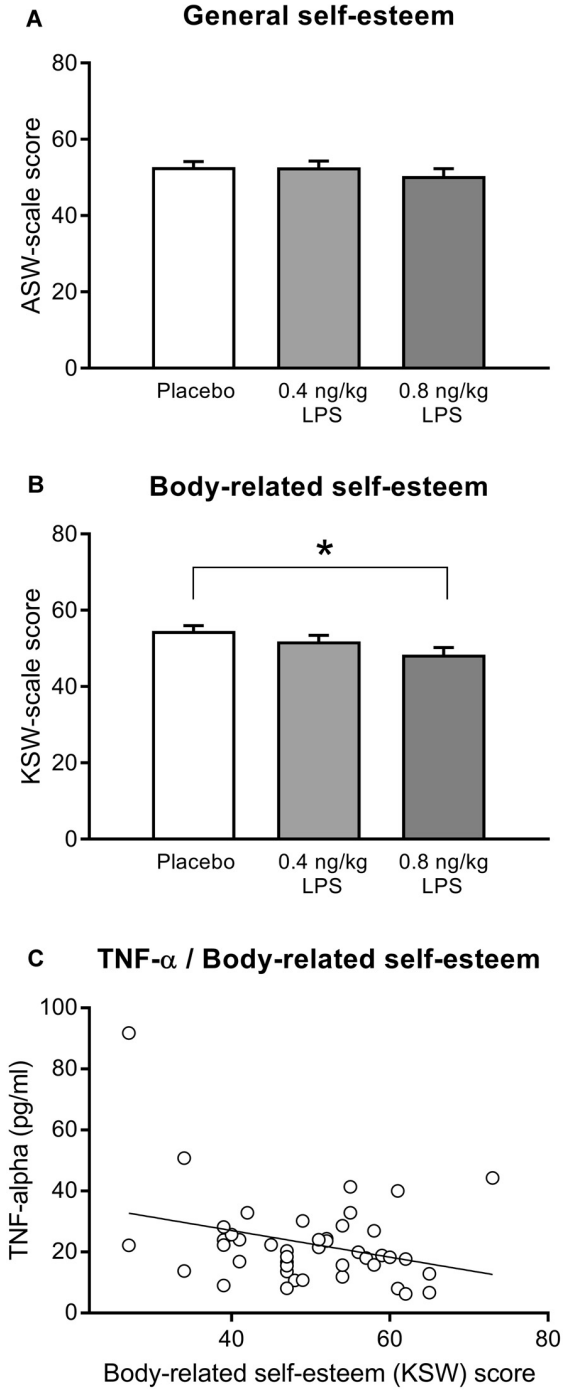

FIGURE 3 | General self-esteem **(A)** and body-related self-esteem **(B)** were assessed with the validated German Multidimensional Self-Worth Scale (MSWS) questionnaire (see text for details) 3 h after injection of either 0.8 or 0.4 ng/kg body weight LPS, or saline (placebo group). Exploratory correlation analysis within the LPS group **(C)** indicated a significant association between body-related self-esteem and TNF-α plasma concentration 3 h after injection ($r = -0.31$, $p < 0.05$; for details, see text). *$p < 0.05$, result of Bonferroni-corrected *post hoc t*-test. For results of ANOVA, see text.

association between body-related self-esteem and inflammatory parameters including IL-6 ($r = -0.23$, $p = 0.13$), CRP ($r = -0.22$, $p = 0.14$), and body temperature ($r = -0.27$, $p = 0.064$) were observed.

Intolerance of Uncertainty (Data Set 1)

Intolerance of uncertainty was assessed with the validated UI-18 questionnaire (**Table 2**). No significant group differences were observed for the three subscales "reduced ability to act" ($F = 1.45$, $p = 0.23$, $\eta_p^2 = 0.03$), "burden" ($F = 1.01$, $p = 0.36$, $\eta_p^2 = 0.02$), or "vigilance" ($F = 0.22$, $p = 0.79$, $\eta_p^2 = 0.01$).

Hopelessness (Data Set 2)

Hopelessness was assessed with the validated H-R-scale. Although LPS-treated participants showed slightly higher hopelessness scores (47.2 ± 1.4) compared to the placebo group (46.1 ± 1.8), no significant difference was found ($F = 0.57$, $p = 0.45$, $\eta_p^2 = 0.01$).

DISCUSSION

Inflammatory processes are increasingly recognized in the pathophysiology of MD (Capuron and Miller, 2004; Schiepers et al., 2005; Raison et al., 2006; Kiecolt-Glaser et al., 2015; Yirmiya et al., 2015; Miller and Raison, 2016; Otte et al., 2016). This is supported by experimental and clinical data suggesting that systemic inflammation contributes to an increased risk of depression (Haroon et al., 2012; Kiecolt-Glaser et al., 2015; Miller and Raison, 2016; Otte et al., 2016). Furthermore, it has been demonstrated that the experimental induction of systemic inflammation in healthy volunteers, e.g., by injecting low-doses of endotoxin, transiently induces dysthymia, anhedonia and fatigue, i.e., symptoms which closely resemble core symptoms of MD (DellaGioia and Hannestad, 2010; Schedlowski et al., 2014). However, whether experimental endotoxemia also impacts neurocognitive processes relevant to the characteristic negative cognitions related to worthlessness, low self-esteem and hopelessness in MD remains unclear. Thus, we herein pooled data from randomized, double-blind, placebo-controlled endotoxin studies in order to assess endotoxin effects on self-esteem, hopelessness and the vulnerability factor intolerance of uncertainty. We observed the expected transient increases in circulating pro-inflammatory cytokines and body temperature, indicating systemic inflammation in response to low-dose LPS. Self-reported positive mood showed a transient decline after LPS application, which is in line with the well-established effects of LPS-induced systemic inflammation on mood (Reichenberg et al., 2001; Wright et al., 2005; Eisenberger et al., 2009; Hannestad et al., 2011; Benson et al., 2017a,b; Engler et al., 2017), and with previous reports documenting an association between dysthymia and LPS-induced increases in cytokine concentrations in plasma (Reichenberg et al., 2001; Eisenberger et al., 2010b) and cerebrospinal fluid (Engler et al., 2017).

Despite the clear and significant LPS-effect on mood, we did not find evidence that LPS induces low self-esteem, hopelessness, or an increased intolerance of uncertainty, i.e., negative thoughts which are common in MD. Interestingly, we observed that body-related self-esteem was slightly, but significantly

TABLE 1 | Sociodemographic and psychological characteristics for data set 1 (upper part) and data set 2 (lower part).

Data set 1	**Placebo (*N* = 38)**	**0.4 ng/kg LPS (*N* = 29)**	**0.8 ng/kg LPS (*N* = 20)**	**Test statistic**	***P***
Age (years)	26.71 ± 1.33	27.24 ± 1.40	26.80 ± 1.07	$F = 0.16$	0.89
Body mass index (kg/m^2)	23.92 ± 0.70	23.08 ± 0.72	23.52 ± 0.57	$F = 0.89$	0.41
Sex (*N*)	*m* = 28, *f* = 10	*m* = 19, *f* = 10	*m* = 20, *f* = 0	/	/
BDI score	3.18 ± 0.87	2.93 ± 0.91	2.90 ± 0.70	$F = 0.07$	0.92
Data set 2	**Placebo (*N* = 34)**		**0.8 ng/kg LPS (*N* = 25)**	**Test statistic**	***P***
Age (years)	26.02 ± 0.96		27.12 ± 0.77	$t = -0.83$	0.40
Body mass index (kg/m^2)	24.32 ± 0.38		23.67 ± 0.47	$t = 1.07$	0.28
BDI score	3.11 ± 0.53		2.64 ± 0.62	$t = 0.58$	0.56

BDI, Beck Depression Inventory. All data are shown as mean ± SEM unless otherwise indicated. m = male, f = female. P = P-value for results of univariate ANOVA (data set 1) or independent samples t-tests (data set 2).

TABLE 2 | Intolerance of uncertainty (data set 1).

UI-18 subscales	**Placebo (*N* = 36)**	**0.4 ng/kg LPS (*N* = 28)**	**0.8 ng/kg LPS (*N* = 20)**	***F***	***P***
Reduced ability to act	10.55 ± 0.94	11.14 ± 0.99	12.00 ± 0.83	1.45	0.23
Burden	13.33 ± 1.16	13.00 ± 1.22	11.55 ± 1.02	1.01	0.36
Vigilance	14.38 ± 1.20	14.00 ± 1.26	13.45 ± 1.06	0.22	0.79

Intolerance of uncertainty was assessed in data set 1 using the validated UI-18 questionnaire (Gerlach et al., 2008). Data from two participants of the placebo group and one participant from the 0.4 ng/kg LPS group are missing. All data are shown as mean ± SEM. P = P-value for results of univariate ANCOVA group effect accounting for baseline BDI scores.

lower in participants who received LPS when compared to the placebo group. This finding suggests that LPS-induced systemic inflammation may transiently impair certain aspects of self-esteem which are related to physical appearance, attractiveness and sportiness rather than affecting self-esteem in general. In detail, the respective subscale comprises items such as "*how often did you feel that other people are more athletically than you?*," or "*how confident are you that other people think you are attractive?*" One likely explanation for the reported reduction in body-related self-esteem is that pro-inflammatory mediators released in response to LPS reportedly induce physical sickness symptoms such as fatigue and pain (Lekander et al., 2016; Benson et al., 2017b). Such symptoms conceivably impact self-perceived physical abilities, and hence the subscale of the questionnaire employed herein. Supporting this notion, we found that lower self-esteem ratings were associated with higher TNF-α concentration. Further, body-related self-esteem was significantly lowered in response to the higher LPS dose only, which mirrors the dose-dependent effects for cytokine concentrations and physical sickness symptoms (Wegner et al., 2014; Benson et al., 2017b). This could also indicate that only higher doses of LPS (and correspondingly higher cytokine concentrations) are capable to induce changes in body-related self-esteem. Moreover, LPS-induced systemic inflammation reportedly affects physical abilities such as walking speed (Sundelin et al., 2015), and even body odor (Olsson et al., 2014), alterations which can be detected by other persons (Regenbogen et al., 2017; Axelsson et al., 2018). Thus, lower ratings of body-related self-esteem may also be related to discrete, but perceivable changes in one's own (outer) appearance during endotoxemia.

Taken together, our data suggest that although experimental endotoxemia effectively induces mood impairments, it does not alter self-referred negative cognitive schemata related to self-worth and hopelessness. Our finding of lower body-related self-esteem during systemic inflammation should be carefully interpreted in the light of its small effect size, which indicates that only a small proportion of the variance in body-related self-esteem was explained by the LPS application. This supports that additional factors including endotoxin-effects on physical sickness symptoms and physical abilities could have contributed to reduced body-related self-esteem. Moreover, it is also conceivable that the transient changes in body-related self-esteem are rather an adaptive and not a maladaptive response to an acute inflammatory event. Lowered body-related self-esteem may encourage reduced physical activity, and may thus contribute to saving energy resources which are needed during an immune activation (Straub, 2017).

Nevertheless, comprehensive evidence from independent studies and groups supports that the LPS model is well-suited to transiently induce other specific features of MD, which are related to mood impairments, social functioning, reward processing, reduced appetite, fatigue, increased pain sensitivity and unspecific physical symptoms (Schedlowski et al., 2014; Kiecolt-Glaser et al., 2015; Miller and Raison, 2016; Lasselin et al., 2018). With respect to cognitive symptoms of MD, it seems most likely that LPS affects cognitive *processes* such as the processing of emotional stimuli (Benson et al., 2017a), but not negative cognitive schemata, which are rather based on learning and repeated experiences (Beck et al., 1979; Bollen et al., 2017). This raises the question if self-referent negative

cognitive schemata could be less responsive to inflammatory processes or pro-inflammatory mediators in general, i.e., if our finding might also apply for patients with MD. It is however also conceivable that negative cognitive schemata, while not responsive to a single and transient immune challenge, could be induced or worsened by repeated inflammatory events or chronic inflammation. Moreover, it is also possible that it is the *interaction* between inflammation and pre-existing vulnerability or situational factors such as psychological stressors, which ultimately affect cognitive schemata in persons at risk.

Our findings should be interpreted in the light of some limitations. Questionnaire data were not available from all primary studies, making it necessary to conduct the analyses in two separate data sets. Moreover, we did not measure cognitive symptoms at BL, making it impossible to control for intra-individual changes in response to LPS-administration. However, cognitive symptoms were assessed in all primary studies at similar time points in LPS- and placebo- groups under strictly standardized conditions. Thus, it seems unlikely that our findings are related to unsystematic differences between study groups. In addition, we can exclude anchor effects, i.e., biased responses due to a recall of BL ratings. We could herein assess only a limited number of inflammatory parameters. Recent research revealed that various peripheral and central cytokines, chemokines, and other molecules are involved in changes of cognitive functioning, including IL-4, Interferon-gamma, CCL-11 and β2-microglobulin (Villeda et al., 2011; Gadani et al., 2012; Smith et al., 2015; Monteiro et al., 2016). Future studies should take these parameters into account when addressing immune-related effects on cognitive functioning. Further, the LPS model allows to induce only a transient immune activation and we could include only young and healthy volunteers with BDI depression scores within a normal range. We therefore cannot conclude about possible associations between inflammatory parameters and self-esteem in individuals with a chronic immune activation and/or pre-existing mood symptoms, e.g., in clinically depressed patients. Thus, one important target for future research would be to assess the effects of immune parameters on negative cognitive schemata in persons at risk for psychiatric symptoms, or in elderly people, who are more prone to chronic inflammation (Zouikr and Karshikoff, 2017). Along the same lines, herein only a small number of female participants could be included in only one data set, which did not allow taking possible sex differences into account. Since only women using different types of hormonal contraceptives were included, it remains open if the results were confounded by different hormonal dosages, and can be translated to free-cycling women. Addressing sex differences and effects of hormonal status would be important given that women showed not only more pronounced responses to immune challenges (Klein, 2012), including LPS (Engler et al., 2016; Lasselin et al., 2018), but also greater LPS-induced changes in depressive symptoms (Moieni et al., 2015b), and a higher prevalence of MD (Hyde et al., 2008).

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

SE, HE, MS and SB contributed to the conception and design of the study. TR, AW and AB collected data. SK and TR organized the database. SK and AB performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version. All authors agreed to be 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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REVIEW
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The High Costs of Low-Grade Inflammation: Persistent Fatigue as a Consequence of Reduced Cellular-Energy Availability and Non-adaptive Energy Expenditure

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Chronic or persistent fatigue is a common, debilitating symptom of several diseases. Persistent fatigue has been associated with low-grade inflammation in several models of fatigue, including cancer-related fatigue and chronic fatigue syndrome. However, it is unclear how low-grade inflammation leads to the experience of fatigue. We here propose a model of an imbalance in energy availability and energy expenditure as a consequence of low-grade inflammation. In this narrative review, we discuss how chronic low-grade inflammation can lead to reduced cellular-energy availability. Low-grade inflammation induces a metabolic switch from energy-efficient oxidative phosphorylation to fast-acting, but less efficient, aerobic glycolytic energy production; increases reactive oxygen species; and reduces insulin sensitivity. These effects result in reduced glucose availability and, thereby, reduced cellular energy. In addition, emerging evidence suggests that chronic low-grade inflammation is associated with increased willingness to exert effort under specific circumstances. Circadian-rhythm changes and sleep disturbances might mediate the effects of inflammation on cellular-energy availability and non-adaptive energy expenditure. In the second part of the review, we present evidence for these metabolic pathways in models of persistent fatigue, focusing on chronic fatigue syndrome and cancer-related fatigue. Most evidence for reduced cellular-energy availability in relation to fatigue comes from studies on chronic fatigue syndrome. While the mechanistic evidence from the cancer-related fatigue literature is still limited, the sparse results point to reduced cellular-energy availability as well. There is also mounting evidence that behavioral-energy expenditure exceeds the reduced cellular-energy availability in patients with persistent fatigue. This suggests that an inability to adjust energy expenditure to available resources might be one mechanism underlying persistent fatigue.

Keywords: cytokines, metabolism, effort, motivation, energy balance, chronic fatigue syndrome, cancer-related fatigue

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INTRODUCTION

Chronic or persistent fatigue is a common, debilitating symptom of several diseases. It is one of the most frequently reported symptoms of cancer and cancer treatment (Servaes et al., 2002; Abrahams et al., 2016) and is highly prevalent in several chronic diseases, such as multiple sclerosis, diabetes, and rheumatoid arthritis (Wolfe et al., 1996; Drivsholm et al., 2005; Induruwa et al., 2012; Sanoobar et al., 2015). In addition, it is the hallmark symptom of chronic fatigue syndrome, a condition in which severe persistent fatigue is experienced in absence of a diagnosed disease (Fukuda et al., 1994; Afari and Buchwald, 2003). Persistent fatigue is distinct from acute fatigue. Acute fatigue is a healthy, adaptive response to physical or mental exertion, inducing metabolic signaling to prevent further energy consumption (Keyser, 2010). Acute fatigue typically resolves after rest or sleep. In contrast, persistent fatigue is often disproportional to exerted activities and is generally not completely alleviated by rest. No treatments for persistent fatigue have been approved by the US Food and Drug Administration, in part because the underlying mechanisms are still poorly understood.

Activation of inflammatory pathways has been suggested to underlie persistent fatigue in many patient populations (Bower, 2014; Karshikoff et al., 2017; Lasselin et al., 2017; Montoya et al., 2017) and animal models (Krzyszton et al., 2008; Mahoney et al., 2013; Bonsall et al., 2015; Norden et al., 2015; Zhang et al., 2016; Vichaya et al., 2017). Indeed, it is well-known from experimental studies that acute severe inflammation, such as induced by lipopolysaccharide (LPS), causes acute sickness behavior, including fatigue. This response has been interpreted as an adaptive process leading to the conservation of energy and reduction of the risk of further dissemination of pathogens (e.g., by withdrawing from social interactions) (Dantzer et al., 2014; Engler et al., 2016). Moreover, in autoimmune diseases such as multiple sclerosis, diabetes, and rheumatoid arthritis, the level of fatigue is associated with an increase in plasma cytokines, especially during symptom relapse (Lasselin et al., 2012; Malekzadeh et al., 2015; Patejdl et al., 2016; Choy and Calabrese, 2017). Associations between small, prolonged increases in plasma inflammatory cytokines and chemokines and persistent fatigue have also been reported in cancer survivors (Bower and Lamkin, 2013) and in individuals with chronic fatigue syndrome (Montoya et al., 2017). In these patients, the levels of plasma cytokines are generally much lower than those detected in patients with autoimmune diseases. The mechanisms by which subtle increases in inflammation induce fatigue are still unclear, and could be different from what has been shown for acute severe inflammation. However, the number of associations reported in the literature suggest that the effect is biologically significant.

We propose that chronic low-grade inflammation induces and/or maintains persistent fatigue by inducing an imbalance between cellular-energy *availability* and cellular- and behavioral-energy *expenditure* (**Figure 1**). Inflammation increases the need of immune cells for rapid generation of cellular energy. To meet this need, immune cells shift to aerobic glycolysis for energy production, a less-efficient, but fast-acting pathway (Kominsky et al., 2010; McGettrick and O'Neill, 2013). During chronic low-grade inflammation, the extended reliance on aerobic glycolysis would be expected to lead to reduced nutrient availability and thus to less energy availability for demanding organ systems. The organismal energy balance can further be encumbered by changes in circadian rhythms and sleep. In addition, there is evidence suggesting that low-grade or chronic inflammation (but not acute severe inflammation) can be linked to increases in *behavioral*-energy expenditure (Vichaya et al., 2014; Lasselin et al., 2017), contributing to the imbalance between energy availability and expenditure and, thereby, leading to fatigue.

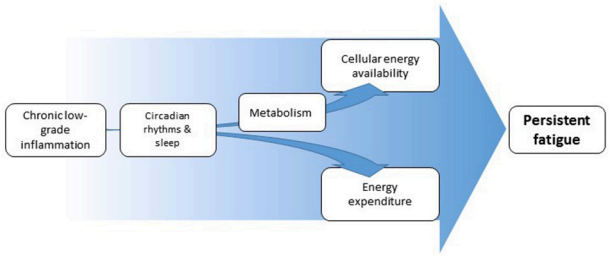

FIGURE 1 | Schematic overview of proposed pathways from inflammation to fatigue. Inflammation affects cellular energy availability through its effects on metabolism. Inflammation can at the same time affect energy expenditure, both through increased energy demand by the immune system and through changes in motivation-driven energy expenditure. Both pathways can be partially mediated by altered circadian rhythms and disturbed sleep. The resulting imbalance in energy availability and expenditure underlies the experience of fatigue.

EFFECTS OF LOW-GRADE INFLAMMATION ON ENERGY PRODUCTION AND EXPENDITURE

Adenosine triphosphate (ATP) is the primary form of energy "currency" utilized by the cells. The generation of ATP involves the catabolism of macronutrients (carbohydrates, lipids, and proteins), each starting from a unique metabolic pathway but ultimately being shuttled to the cells for ATP production (**Figure 2**). Factors that can negatively affect ATP production include reduced intracellular glucose availability through either alterations in macronutrient metabolism or reduced glucose uptake by the cells; reduced functioning of the mitochondria for aerobic energy production and subsequent increased dependence on less-efficient aerobic glycolysis.

Energy Production During Prolonged Inflammation

Inflammation requires a change in metabolism, and these changes differ between acute and chronic or prolonged

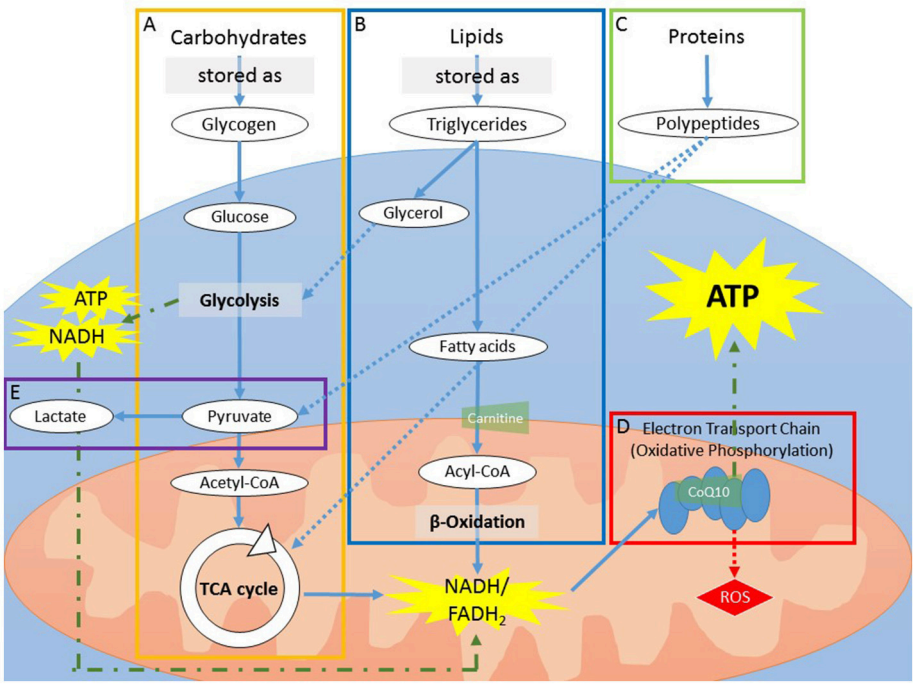

FIGURE 2 | (A) Carbohydrates can be stored as glycogen, which can be rapidly utilized for fast energy production. As storage is limited to a handful of organs, mainly the liver and skeletal muscles, sustained usage of glycogen will deplete stores in a few h. During catabolism, glycogen is first broken down into glucose molecules that enter into glycolysis, yielding pyruvate. The process of glycolysis yields a low amount of ATP and NADH. After the addition of a CoA group, pyruvate (now acetyl-CoA) enters into the TCA cycle to produce NADH and $FADH_2$. **(B)** In contrast to carbohydrate storage, storage of lipids, in the form of triglycerides, is virtually limitless. Catabolism of lipids is a slow process and is therefore mainly utilized during prolonged energy need (i.e., when carbohydrate storage is expended). The process yields fatty acids and glycerol. The addition of a CoA group to fatty acids generates acyl-CoA, which is carried into the mitochondria via the carrier protein carnitine. Once inside the mitochondria, acyl-CoA is catabolized via β-oxidation to produce NADH and $FADH_2$. Glycerol can enter into the end steps of glycolysis or is reprocessed to form glucose (i.e., gluconeogenesis). **(C)** Proteins can be broken down in smaller polypeptides or amino acids during ATP production but cannot be stored. As proteins are required for biological functions other than ATP production, they are thought to be used for ATP production only in conditions of extreme demand, such as sickness or chronic inflammation. Proteins used for ATP production do not go through glycolysis, but instead are converted to TCA metabolites or pyruvate. **(D)** NADH and $FADH_2$ generated by glycolysis, β-oxidation, and the TCA cycle are converted via the electron transport chain (ETC) in the mitochondria. The ETC is a series of 5 protein complexes that work in synchrony to produce ATP. This process, called oxidative phosphorylation, requires oxygen. CoQ10 functions as an electron carrier between the complexes of the electron transport chain. **(E)** In the absence of oxygen, or when mitochondria are impaired, glycolysis is the dominant energy-producing metabolic pathway. In order for glycolysis to continue, however, NAD+, a secondary substrate along with glucose, must be regenerated from NADH. To do so, pyruvate is converted into lactate, an energy-requiring mechanism that utilizes NADH and thus decreases overall ATP production. ATP, adenosine triphosphate; CoA, coenzyme A; CoQ10, coenzyme Q10; ETC, Electron transport chain; $FADH_2$, flavin adenine dinucleotide; NADH and NAD+, forms of nicotinamide adenine dinucleotide; TCA, tricarboxylic acid.

inflammation (**Figure 3**). During acute inflammation, rapid ATP production in immune cells is required for the multitude of immune responses. Immune cells (lymphocytes and leukocytes) switch from oxidative phosphorylation to an increased reliance on aerobic glycolysis for rapid ATP production (Kominsky et al., 2010; McGettrick and O'Neill, 2013; Kelly and O'Neill, 2015). This shift is in favor of precipitous ATP production while catabolic efficiency is sacrificed, resulting in decreased mitochondrial function and increased lactate production.

Additionally, prolonged inflammation is associated with increased insulin resistance and reduced glucose tolerance, leading to reduced glucose uptake by the immune cells and, consequently, less overall ATP production from glucose (Shoelson et al., 2006; Asghar and Sheikh, 2017). Therefore, during ongoing low-grade inflammation, energy and glucose are diverted from other systems to support the immune response. To compensate, the body increases lipid and protein metabolism for ATP production (Liu et al., 2012c). Lipid metabolism is a slow process and thus cannot respond to rapid energy requirements. Additionally, increasing reliance on protein metabolism means other functions, such as promotion of growth stimulation, are compromised (Le Floc'h et al., 2004). Inflammation also leads to increased reactive oxygen species (ROS) to combat invading pathogens (Liu et al., 2012a; Maitra et al., 2012). However, ROS can be harmful to mitochondria and mitochondrial DNA (Sinha et al., 2013) and thus, prolonged exposure to ROS during chronic inflammation contributes to mitochondrial dysfunction.

The effects of chronic inflammation are especially detrimental for neurons that heavily depend on oxidative phosphorylation for their ATP needs (Hall et al., 2012). In normal brain metabolism, neurons rely heavily on astrocytes. Astrocytes use glycolysis to convert lipids and glucose to pyruvate and then lactate. Lactate is

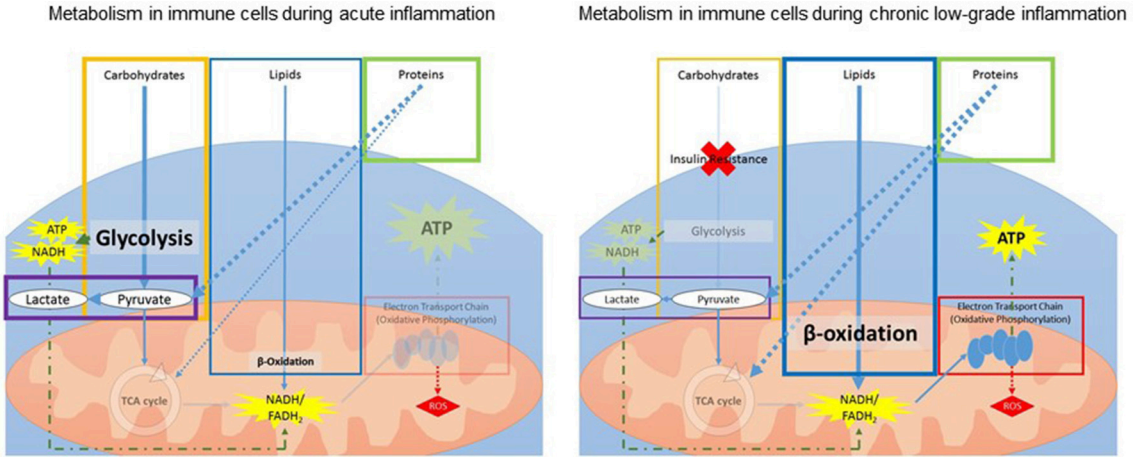

FIGURE 3 | Schematic overview of changes in metabolism within immune cells during acute **(Left)** and chronic **(Right)** inflammation. During acute inflammation, immune cells increase glycolysis while decreasing the TCA cycle for fast generation of ATP. During chronic *low-grade* inflammation, insulin resistance leads to a decrease in glucose uptake and glycolysis. To compensate, the body increases lipid and protein metabolism for ATP production. ATP, adenosine triphosphate; $FADH_2$, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; TCA, tricarboxylic acid.

then shuttled to neurons where it is enters the tricarboxylic acid (TCA) cycle. During chronic inflammation, insulin resistance decreases astrocytic glucose and protein metabolism, resulting in reduced lactate availability for neurons (Blázquez et al., 2014), forcing the neurons to rely more heavily on the slower process of lipid metabolism. Further, inflammation is able to low mitochondrial efficiency (Yan et al., 2013), increasing the metabolic burden on neurons. Alterations in neuronal metabolism will ultimately affect neuronal functioning.

Behavioral-Energy Expenditure During Inflammation

Chronic low-grade inflammation has been estimated to increase systemic energy expenditure by up to 10% (Straub, 2017). Given the increased energy demand of chronic inflammation, and the resulting reduced energy availability, one would expect that *behavioral*-energy expenditure (i.e., amount of energy spent on activities) would decrease. Several studies in the context of acute, severe inflammation corroborate this hypothesis showing reductions in willingness (motivation) to exert effort, both in rodent models (Larson et al., 2002; Felger et al., 2013; Nunes et al., 2014; Yohn et al., 2016) and in humans (Draper et al., 2017) (**Table 1**). However, results from these and other studies also suggest that motivational behavior might be differentially affected in conditions of low-grade inflammation.

Studies on animal models show a reduction in effort expenditure for food only after administration of higher doses of inflammatory mediators (Larson et al., 2002; Felger et al., 2013; Nunes et al., 2014; Yohn et al., 2016). For the lowest doses, not only was there no reduction in activity, there also seemed to be a trend for increased activity (Larson et al., 2002). Further, Vichaya et al. (2014) tested animals 24 h after an acute inflammatory insult (LPS injection), at which point the acute inflammatory response and sickness behavior had subsided. A shift was observed in motivational priority *toward* choices that required a higher effort for a more salient reward.

Comparing the results from the two available studies in humans also point to distinct effects of high vs. low-grade inflammation on effort expenditure choices. Draper et al. (2017) tested healthy individuals 2 and 5 h after LPS/saline injection and found a reduced willingness to perform high-effort tasks in LPS-treated participants at 2, but not 5, h post injection. At 2 h, inflammation was as its peak. In contrast, Lasselin et al. (2017) tested participants 5 h post LPS/saline injection and observed an *increased* willingness to perform high effort tasks in LPS-treated participants (but only when the conditions to win were favorable). Lasselin et al. further noted that participants were too weak to be tested between 3 and 4 h post injection. Of note, the two studies differed in several important aspects including sample characteristics (only males vs. mix of males and females) and task design (choice between accepting or rejecting a task vs. choice between a high effort/high reward or a low effort/low reward task) (see **Table 1** for study specifics). Nevertheless, we hypothesize on the basis of these human and murine experimental data that acute severe inflammation leads to overall adaptive *reductions* in effort expenditure, whereas mild inflammation can lead to relative *increases* in effort expenditure provided the incentive is strong enough. This notion is supported by our findings that low-grade inflammation was associated with *increased* high-effort choices in a sample of cancer patients and survivors (Lacourt et al., under review). It is still to be determined if these behavioral shifts in effort expenditure correspond to "recovery" or to a switch in immune cells back from a glycolytic to a more oxidative-driven metabolic profile. In addition, behavioral shifts during *chronic* low-grade inflammation have not yet been studied.

The effects of inflammation on effort expenditure in motivational tasks appear to be mediated by the dopaminergic system (Felger et al., 2013; Yohn et al., 2016). Although

TABLE 1 | Overview of discussed studies on the effects of inflammation on effort expenditure.

References	Study description	Study targets	Main results on motivational effort expenditure
ANIMAL MODELS			
Larson et al., 2002	Mice tested in an operant conditioning paradigm 90 min after IL-1β or saline control. Testing included willingness to perform nose pokes for a reward (sweetened milk) under fixed ration schedules (FR; fixed number of nose pokes needed for reward) and progressive ratio schedules (PR; number of required nose pokes increases incrementally with each reward).	Comparison of saline vs. IL-1β (30, 100, and 300 ng). Outcome was number of completed trials within a given time (response rate) on FR4, 10, or 32 or maximal number of responses made for a single pellet (breaking point) on a PR10 schedule.	100 and 300 ng IL-1β led to decreased response rate on the FR32 and to a significant decrease in the breaking point on the PR10. *30 ng led to slight (nonsignificant) increases in response rate in the FR10 and in break point in the PR10, suggestive of increased willingness to exert effort under low-grade inflammation.*
Felger et al., 2013	Rhesus monkeys were tested after 4 weeks of saline vs. IFN-α treatment (administered 5 days per week to mimic monotherapy for malignant melanoma) in a randomized repeated measures design.	Comparison of saline vs. IFN-α treatment on willingness to work for a sucrose treat. Outcome was the number of sucrose pellets obtained from a puzzle feeder (requiring work to obtain the treat).	IFN-α treatment led to a reduction in sucrose pellets obtained from the puzzle feeder. Consumption of pellets from the regular feeder was not reduced by IFN-α, suggesting no change in anorexia or appetitive behavior.
Nunes et al., 2014	Rats were tested in an FR5-lever pressing protocol 90 min after saline or IL-1β in the presence of freely available but less preferable regular chow.	Saline vs. IL-1β (1.0, 2.0, and 4.0 mg/kg). Outcomes were response rates on the FR5 and amount of freely available chow consumed.	IL-1β at 2.0 and 4.0 mg/kg dose decreased response rates compared to saline. This was paired with significant increases in consumption of freely available regular chow, suggesting that the effects of IL-1β were not due to change in appetitive behavior. No effect was observed for the 1.0 mg/kg dose of IL-1β.
Yohn et al., 2016	Rats were tested in an FR5-lever pressing protocol 45 min after saline or IL-6 administration in the presence of freely available but less preferable regular chow.	Saline vs. IL-6 (2.0, 4.0, 6.0, and 8.0 mg/kg). Outcomes were response rate on the FR5 and amount of freely available chow consumed.	IL-6 at 4.0–8.0 mg/kg dose led to decreased response rate compared to saline. Microdialysis revealed reduced extracellular dopamine in the accumbens following IL-6 administration.
Vichaya et al., 2014	Mice tested approximately 24 h after LPS or saline on a concurrent choice operant conditioning task (FR-10 for preferred chocolate pellets and FR-1 for a less-preferred grain pellet).	Saline vs. LPS (0.33 mg/kg). Outcome was total number or nose pokes for chocolate and grain rewards as well as chocolate preference (% chocolate pellets earned).	While LPS led to a reduction in total number of nose pokes, this shift was mostly driven by a reduction in nose pokes for grain, resulting in an increase in percentage of chocolate pellets after LPS.
CLINICAL STUDIES			
Lasselin et al., 2017	Healthy subjects ($n = 21$; 9 women) tested on effort expenditure 5 h after LPS vs. saline injection in a cross-over design.	LPS (2 ng/kg) vs. saline. Outcome was ratio of high effort-high reward/low effort-low reward choices under different reward conditions.	Subjects showed an increase in ratio of high effort choices after LPS, only when reward conditions were more preferable. The effect of LPS was mediated by increased sleepiness. *Subjects were tested 5 h after injection at which time plasma IL-6 concentrations and acute sickness symptoms had started to decrease in the LPS condition.*
Draper et al., 2017	Healthy subjects ($n = 29$; all male) tested on effort expenditure choices 2 and 5 h after LPS vs. saline injection in a cross-over design.	LPS (2 ng/kg) vs. saline. Outcome was number of accepted offers to exert effort (squeezing a dynamometer) for a reward under different reward conditions.	Subjects accepted fewer offers with high effort requests 2 h after LPS. No effect of LPS was found after 5 h. *At 2 h post injection IL-6 and TNF-α concentration were at their peak, but sickness symptoms were decreased. No effects on effort expenditure were found 5 h post LPS, at which time IL-6 and TNF-a levels had almost returned to control levels.*

the mechanism by which inflammation is capable of altering dopamine neurotransmission is still unclear, the literature on Parkinson's disease indicates that chronic neuroinflammation leads to oxidative stress and mitochondrial damage in dopaminergic neurons (Niranjan, 2014; Blesa et al., 2015), leading to an impairment in dopaminergic neurotransmission. Dopaminergic neurons are particularly vulnerable to inflammation (de Pablos et al., 2014; Park et al., 2016). Although the cause of this vulnerability is not fully understood, the higher basal oxidative phosphorylation of these cells and the auto-oxidation of excess extracellular dopamine may contribute (Asanuma et al., 2003; Pacelli et al., 2015).

Sleep and Circadian Rhythms as Partial Mediators of the Effects of Chronic Low-Grade Inflammation on Energy Availability and Expenditure

Inflammation can additionally lead to more-sustained alterations in metabolism via changes in circadian rhythms and sleep. Chronic low-grade and severe inflammation has been shown to affect sleep quality and circadian rhythms (Pollmächer et al., 2000; Haspel et al., 2014; Anderson et al., 2015), and vice versa (Leproult et al., 2014; Rahman et al., 2015; Wright et al., 2015). Alterations in circadian activity rhythms have been linked to fatigue (Payne, 2011; McHill and Wright, 2017). Sleep disturbances or reduced sleep quality—often reported by individuals with persistent fatigue—can also lead to disorganized or misaligned circadian rhythm through daytime napping and structural alterations in nighttime sleep onset.

Most metabolic processes are regulated by the circadian clock, and changes in circadian rhythm or sleep are associated with metabolic changes, such as increased circulating glucose and decreased insulin sensitivity (Depner et al., 2014; Potter et al., 2016). Specifically, expression of mitochondrial proteins involved in many metabolic processes undergo posttranslational modifications regulated by the *Clock* gene (Masri et al., 2013). In addition, mitochondrial oxidative metabolism is also controlled by the circadian clock through oscillations in biosynthesis of nicotinamide adenine dinucleotide (NAD+) and availability of rate-limiting mitochondrial enzymes (Peek et al., 2013; Neufeld-Cohen et al., 2016).

Insufficient sleep and changes in circadian rhythms can also alter cellular-energy and behavioral-energy expenditure. Preclinical studies show increased energy expenditure after total sleep deprivation in rats (Caron and Stephenson, 2010). Likewise, experimentally induced sleep deprivation in healthy human volunteers led to increased daily energy expenditure, assessed via whole-room calorimetry (oxygen consumption and carbon dioxide production). Although this was accompanied by a compensatory increase in energy (food) intake (i.e., caloric intake exceeded caloric expenditure), physical exhaustion increased significantly (Markwald et al., 2013). As the above described studies rely on experimentally induced alterations in circadian rhythm and sleep, it is unclear to what extent these findings are relevant for naturally occurring changes. In addition, whether more subtle, sustained changes in sleep and circadian rhythm, such as observed in fatigued individuals, also alter energy expenditure remains to be shown.

Summary of Proposed Pathways

Chronic low-grade inflammation is related to a persistent metabolic switch to faster, but less efficient, glycolytic energy production, and increased ROS production. This in its turn affects mitochondrial function, thereby increasing the reliance on glycolytic pathways. In addition, although chronic inflammatory processes demand more energy, this increased need for energy is not always accompanied by decreases in behavioral-energy expenditure. This seems particularly the case in the context of low-grade inflammation, which has been shown to increase motivation for effort expenditure. Inflammation can also affect circadian rhythms and sleep (and vice versa), which can further exacerbate the effects on energy availability and expenditure.

We propose that this imbalance of energy availability vs. expenditure underlies the experience of fatigue induced by chronic low-grade inflammation. Below, we discuss the evidence for these metabolic pathways in persistent fatigue.

PERSISTENT FATIGUE AS A RESULT OF REDUCED ENERGY AVAILABILITY AND INCREASED ENERGY EXPENDITURE?

Associations between low-grade inflammation and persistent fatigue have been reported predominantly for individuals with CFS or CRF. In patients with CFS, fatigue severity has been associated with elevated cytokine concentrations in plasma (e.g., Montoya et al., 2017) and spinal fluid samples (e.g., Hornig et al., 2016). Alterations in physiological stress-response (predominantly hypothalamic-pituitary-adrenal axis output) in patients with CFS have also been reported (e.g., Silverman et al., 2010), suggesting that low-grade inflammation might be the result of reduced sensitivity to immune-regulating stress hormones such as cortisol. For CRF, fatigue usually starts during cancer treatment but persists into survivorship in a significant subset of patients (Servaes et al., 2002; Goedendorp et al., 2013). Most first-line treatments such as chemotherapy and surgery are known to induce an inflammatory response (e.g., due to tissue damage) (Fitzpatrick and Wheeler, 2003; Boomsma et al., 2010; Wang et al., 2016). Increases in inflammatory markers have indeed been associated with increased fatigue severity in patients actively undergoing treatment, although not all inflammatory markers associated with fatigue show a response to cancer treatment, suggesting the presence of other causal factors (Reed et al., 2016). In cancer survivors, despite the cessation of the immediate inflammatory effects of cancer treatment, associations between elevated levels of inflammatory markers and increased fatigue have also been observed (Bower, 2007). CRF in survivors has in addition been associated with psychosocial stress factors, such as fear of cancer recurrence (Young and White, 2006), suggesting that inflammatory processes during survivorship might (in part) be stress-related (Lacourt and Heijnen, 2017).

BOX 1 | Mitochondria and biomarkers of mitochondrial function.

Healthy mitochondrial functioning depends on a multitude of factors. Mitochondria themselves produce reactive oxygen species (ROS) as a byproduct of metabolism. In normal conditions, antioxidants are sufficient at protecting against damage from ROS, and the cell remains in equilibrium. However, if the balance skews toward ROS production during excessive and/or dysfunctional mitochondrial activity, ROS can induce the production of several toxins and can damage the cell; this is referred to as oxidative stress. Most patient studies on biomarkers of mitochondrial function have focused on circulating levels of coenzyme Q10 (CoQ10) or *L*-carnitine. **CoQ10** is a component of the electron transport chain of the mitochondria and is thus important for mitochondrial energy production. It is assumed that CoQ10 serum levels mirror levels in the mitochondria, although to the best or our knowledge this has not been formally studied. The antioxidant properties of CoQ10 make it an anti-inflammatory agent as well as an important mitochondrial protectant (Genova et al., 2003; Sanoobar et al., 2015). *In vitro* and animal studies have indeed shown a beneficial effect of CoQ10 on mitochondria (Bergamini et al., 2012; Jiménez-Santos et al., 2014).
***L*-carnitine** can be either made by the body or acquired in the diet. L-carnitine is important for transport of fatty acids across the mitochondrial membrane for β-oxidation and adenosine triphosphate production. Thus, reductions in L-carnitine indicate reduced availability of fatty acids in the mitochondria and reduced tricarboxylic acid activity (Porter et al., 2017).

Reduced Energy Availability in Persistent Fatigue

Evidence of reduced energy availability in persistent fatigue comes mainly from metabolomic studies (e.g., Fluge et al., 2016; Naviaux et al., 2016; Yamano et al., 2016) and from studies of mitochondrial dysfunction (e.g., Filler et al., 2014).

Mitochondrial Dysfunction

Mitochondria are often represented as the "powerhouses of the cell." Mitochondrial function can be compromised by a multitude of factors, including reduced availability of necessary metabolites and mitochondrial damage through, for example, oxidative stress (**Box 1**). The notion that mitochondrial dysfunction can lead to fatigue is indicated by the observation that fatigue is a common symptom of mitochondrial disease (Gorman et al., 2015) and that fatigue-like behavior in animal models is associated with reduced expression of mitochondrial complexes, reductions in metabolic activity, and alterations in mitochondrial morphology in the brain (Surapaneni et al., 2012; Wang et al., 2014; Vichaya et al., 2016).

The association between mitochondrial dysfunction and persistent fatigue has been most widely studied in patients with CFS (**Table 2**). A systematic review reported that 21 of 25 papers were restricted to CFS (Filler et al., 2014). Lower serum levels of the antioxidant CoQ10 was the most consistent finding of the review, with limited evidence for lower levels of other antioxidants and increased levels of oxidative stress markers. Lower carnitine levels were observed in some studies, but results depended on the type of carnitine assessed. Later published studies on CFS confirm increased oxidative stress levels, possible decreases in ATP production efficiency, and reductions in mitochondrial energy production in patients with CFS (Ciregia et al., 2016; Tomas et al., 2017). The assessment of oxygen consumption in PBMCs by Tomas et al. (2017) is promising, indicating a reduced capacity of mitochondria to meet increased energy demands during cellular stress, a replication is warranted based on the use of both frozen and fresh blood samples and the limited sample size.

Studies on CRF have thus far been limited to the immediate effects of cancer treatment, and it is unclear to what extent these effects are still present into survivorship. Preclinical research has shown that both cancer and cancer therapy are associated with mitochondrial dysfunction (Dumas et al., 2011; Tzika et al., 2013; Luo et al., 2014; Gouspillou et al., 2015; Sridharan et al., 2015; Tabassum et al., 2015; Chiu et al., 2016; Gilliam et al., 2016; Vichaya et al., 2016). It is unknown from preclinical models whether treatment-induced or cancer-induced changes in mitochondrial function are associated with fatigue. However, a handful of observational patient studies from one research group report an association in prostate cancer patients undergoing radiation therapy. Their results point to reduced mitochondrial functioning in association with fatigue, as evidenced by reduced expression of mitochondria-related genes (Hsiao et al., 2013, 2014), decreased expression of mitochondrial electron transport complex II enzymes (Filler et al., 2016), and increased apoliprotein A1 (ApoA1) in association with higher fatigue (Lukkahatai et al., 2014). ApoA1 is important for lipid scavenging, and an increase in ApoA1 is indicative of a reduced use of lipids for ATP synthesis.

A handful of studies and clinical trials corroborate the above-described observational findings. Two experimental studies in patients with CFS showed alterations in metabolic processes. Results of *in vitro* stimulation of skeletal muscle cells from patients with CFS were indicative of insulin resistance or decreased sensitivity of insulin receptors (Brown et al., 2015). Further, patients with CFS had a slower recovery from an initial maximal exercise test, leading to a more rapid reliance on less-efficient glycolytic metabolism during a subsequent test (Snell et al., 2013).

Antioxidant supplementation can be effective in reducing fatigue, suggesting a causal relationship between reduced availability of antioxidants and fatigue. In patients with CFS, supplementation with CoQ10 and nicotinamide adenine dinucleotide (NADH) reduced fatigue, whereas a placebo treatment did not (Castro-Marrero et al., 2014). Supplementation has also been shown to improve CRF. Cachexic patients with advanced-stage gynecological cancer seemed to benefit from the addition of several metabolism-related supplements to their standard cachexia treatment with synthetic progestogen, showing decreases in fatigue, inflammation, and resting-state energy expenditure as compared to standard treatment alone (MacCiò et al., 2012). In breast cancer patients undergoing chemotherapy, supplementation with an amino-acid jelly that contained CoQ10 and L-carnitine led to less-severe fatigue (Iwase et al., 2016). As this study included a standard-of-care condition as control, a placebo effect cannot be ruled out. In contrast, a large study of breast cancer patients undergoing chemotherapy using supplementation of vitamin E with or without CoQ10 did not find any effect of the added CoQ10 on fatigue development (Lesser et al., 2013). In animal models, it has also been shown that

TABLE 2 | Overview of discussed studies on the association between fatigue and cellular energy production.

References	Study design and sample description	Description	Relevant results
MITOCHONDRIAL FUNCTIONING—OBSERVATIONAL STUDIES			
CFS			
Filler et al., 2014	Review; 25 papers of which 20 included patients with CFS/ME, which are summarized here.	Description of studies assessing associations between fatigue and outcomes of mitochondrial function.	Most consistent evidence for lower serum levels of CoQ10 in patients with CFS (4/4 studies). Other promising findings included reduced carnitine levels (4/5 studies); decreased antioxidant levels (2/2 studies); changes in mitochondrial structure (3/4 studies); and impaired energy production (2/4 studies).
Ciregia et al., 2016	Cross-sectional study; Twins discordant for CFS; Further validation in a sample of patients with CFS ($n = 45$) and healthy controls ($n = 45$).	Proteomic analysis of platelet-derived mitochondria.	Initial and validation analyses showed upregulation in aconitate hydratase (ACON) and ATP synthase subunit beta (ATPB). *ACON is a biomarker for increased oxidative stress and ATPB is associated with ATP production. The authors suggest that the observed upregulation in ATPB indicates an attempt to increase ATP production due to ATP production inefficiency.*
Tomas et al., 2017	Cross-sectional study; Patients with CFS ($n = 63$) and controls ($n = 15$).	Oxygen consumption and glycolytic activity in PBMCs (fresh or frozen).	Samples from CFS patients showed reductions in basal respiration, proton leak, maximal respiration, and spare capacity. No differences were found in glycolytic activity. *Fresh samples were available for only 3 controls and strong differences were observed between fresh and frozen samples, reducing the reliability of the findings. Strongest effect was found for reductions in maximum capacity.*
CRF			
Hsiao et al., 2013	Longitudinal observational study; Non-metastatic prostate cancer patients (NMPC) ($n = 15$); Healthy controls ($n = 15$) were included for reference values.	Change in mitochondria-related gene expression in peripheral blood samples in association with change in fatigue during external beam radiation therapy (EBRT).	Gene expression and fatigue severity did not differ at baseline between patients and controls. In patients, fatigue increased during EBRT. Of the 11 genes that were differentially expressed during EBRT (as compared to baseline), 8 were significantly associated with fatigue scores during radiation. Upregulated genes: BCL-2, FIS1, SLC25A37; downregulated genes: AIFM2, IMMP2L, MSTO1, SLC25A23, and SLC25A24.
Hsiao et al., 2014	Longitudinal observational study; NMPC patients ($n = 25$) undergoing (+EBRT); NMPC patients on active surveillance ($n = 25$) were included as controls (−EBRT).	Changes in expression of 168 mitochondria-related genes in peripheral blood samples in association with fatigue during EBRT.	Patients +EBRT and -EBRT did not differ in fatigue severity or gene expression at baseline. Patients +EBRT showed increased fatigue during treatment. Out of 14 genes that were differentially expressed during EBRT (as compared to baseline), 4 genes were associated with fatigue severity at baseline and during EBRT. Increased fatigue – downregulation of gene: BCL2LI, SLC25A37, FIS1; increased fatigue – upregulation of gene: BCS1L. Confirmatory protein expression analyses showed no associations between fatigue scores and gene-related protein concentrations.

(Continued)

TABLE 2 | Continued

References	Study design and sample description	Description	Relevant results
Lukkahatai et al., 2014	Longitudinal observational study; NMPC patients undergoing EBRT ($n = 12$).	Serum proteomic profile before and midway through EBRT (day 21).	Apolipoprotein A1 (Apo1), ApoE, and transthyretin (TTR) were identified to have changed between baseline and day 21. Patients were post hoc divided into high fatigue (n=9; higher fatigue during EBRT) and no fatigue (n=3) based on their fatigue at day 21. The group characterized as 'high fatigue' had higher ApoA1 expression at day 21 but not at baseline and showed an increase in ApoE during treatment, whereas the 'low fatigue' group did not. TTR values did not differ between groups and did not change significantly in either group.
Filler et al., 2016	Longitudinal observational study; NMPC patients ($n = 22$) undergoing EBRT.	Expression of enzymes of mitochondrial oxidative phosphorylation complexes (complexes I-V) and the antioxidant Manganese superoxide dismutase (MnSOD) in serum in association with changes in fatigue between pre-EBRT and the last day of EBRT.	Lower expression of Complex II enzymes were associated with decreased fatigue scores at baseline and at completion of EBRT. *Post-hoc* characterization of patients as high fatigue and low fatigue showed that the between complex II enzymes and fatigue was only observed in the 'high fatigue' group. Enzyme levels for every complex showed an increase in the high fatigue group and a decrease in the low fatigue group; these within-subgroup changes were not significant. MnSOD did not change significantly in either group.
EXPERIMENTAL STUDIES AND CLINICAL TRIALS			
CFS			
Brown et al., 2015	*In vitro* study of skeletal muscle cells cultures; Cells obtained from patients with CFS ($n = 10$) and age-matched controls ($n = 7$).	Effects of electrical pulse stimulation (EPS) of skeletal muscle cells.	EPS led to insulin-stimulated glucose uptake in control samples but not in CFS samples. EPS-induced IL-6 secretion was seen in samples from both groups but overall IL-6 secretion was lower in the CFS samples. Both groups showed a similar increase in lactate dehydrogenase in response to EPS.
Snell et al., 2013	Experimental study; Patients with CFS ($n = 51$) and controls ($n = 10$).	Physiological responses to repeated maximal exercise tests	Patients with CFS reached their ventilator threshold (VT) at a lower workload during the second exercise test while controls did not show a change in workload at VT.
Castro-Marrero et al., 2014	Randomized controlled clinical trial; Patients with CFS ($n = 73$).	Effects of 8-week oral CoQ10 and NADH supplementation vs. placebo on fatigue and metabolic outcomes.	Supplementation led to a reduction in fatigue and increased PBMC levels of NAD+, ATP, CoQ10, and citrate synthase activity as well as lower NADH and lipid peroxidation. No changes were observed in the placebo group.
CRF			
MacCiò et al., 2012	Randomized controlled clinical trial; Advanced-stage gynecological cancer patients with cachexia ($n = 124$).	Evaluating the effects of 4-month treatment with either synthetic progestogen alone (standard cachexia treatment) or with addition of L-carnitine, celecoxib, and antioxidants.	Additional supplementation led to stronger decreases in fatigue, resting-state energy expenditure (indirect calorimeter), IL-6 and TNF-a concentrations, and ROS.
Iwase et al., 2016	Open label clinical trial; Breast cancer patients undergoing chemotherapy.	Effects of 21-day supplementation with amino-acid jelly containing CoQ10 and L-carnitine or standard-of-care on reported fatigue.	Patients reported less-severe fatigue after supplementation.

(Continued)

TABLE 2 | Continued

References	Study design and sample description	Description	Relevant results
Lesser et al., 2013	Randomized controlled clinical trial; Breast cancer patients planned for adjuvant chemotherapy ($n = 236$).	Effects of 24-weeks supplementation with vitamin E ± CoQ1 on reported fatigue.	Supplementation increased plasma CoQ10 levels, but did not affect fatigue outcomes.
ANIMAL MODELS			
Davis et al., 2009	Mice were required to run to exhaustion on a treadmill or were provided access to a voluntary wheel.	Evaluated effect of 12.5 or 25 mg/kg quercetin (antioxidant/ anti-inflammatory) via oral gavage for 7 days prior to treadmill test. For voluntary wheel running mice were supplemented in their food.	Both doses of quercetin increased maximal endurance capacity and the 25 mg/kg dose increased voluntary wheel running activity. Further, both doses increased PGC1α, SIRT1, and cytochromie c in the brain and soleus muscle. Only the 25 mg/kg dose increased brain and soleus mtDNA copy number.
Fu et al., 2010	Mice were subjected to weight-loaded forced swim for 30 min after the final drug treatment.	Evaluated effect of CoQ10 (0, 1.5, 15, or 45 mg/kg/day for 4 weeks) on fatigue-like behavior.	The 15 mg/kg/day dose of CoQ10 increased swim time to exhaustion. CoQ10 also decreased urea nitrogen post-exercise, increased pre-exercise glycogen (at 15 and 45 mg/kg doses), and had no significant impact on lactic acid.
Singh et al., 2002a	Mice subjected to forced swim (6 min/day for 7 days) as a model of CFS	Evaluated effects of concurrent administration of various agents [i.e., (alleged) anti-oxidants GS-02, melatonin, carvedilol, and St. Johns wort; antidepressant fluoxetine].	Carvedilol, melatonin, St. Johns wort, and GS-02 all reduced immobility from days 2 to 7. Fluoxetine reduced immobility in the swim test on days 1–2, but had no effect on days 3–7. Further, antioxidant treatment, but not fluoxetine, reduced brain enzyme levels of MDA and catalase while increasing GSH and SOD levels.
Singh et al., 2002b	Mice subjected to forced swim (6 min/day for 15 days) as a model of CFS	Evaluated antioxidant effects of various agents [i.e., (alleged) antioxidants withania somnifera root extract, quercetine, melatonin, carvedilol, and St. Johns wort].	As described above, the authors report beneficial effects on immobility time from melatonin, carvedilol, and St Johns wort. Quercetine and withania somnifera also showed protective effects, with the least immobility shown in the withania somifera group. All groups showed reductions in brain MDA.
Surapaneni et al., 2012	Forced swim (15 min/day for 21 days) as a model of CFS in rats	Evaluated behavior and measures of mitochondria function in control mice and those supplemented during the 21 days with withania somnifera and shilajit.	Forced swimming increased immobility during swimming, enhanced anxiety-like behavior, reduced mitochondrial membrane potential, reduced mitochondrial parameters (e.g., NADH, SDH, Cyto c oxidase, ATP synthase). These effects were attenuated by withania somnifera and shitajit.
Vichaya et al., 2016	Mouse model of cancer and cancer-therapy (cisplatin + leg radiation)	Described cancer and therapy induced behavioral changes (burrowing) and brain and liver mitochondria complex gene expression.	The most profound effect on behavior and brain mitochondria complex gene expression was in the tumor-bearing mice treated with cancer therapy. (Note that liver mitochondrial complex gene expression was most effected in the tumor-untreated group.)
Wang et al., 2014	Forced swim (6 min/day for 15 days) as a model of CFS in mice	Evaluated the antioxidant effects of polysaccharides from Panax ginseng (WGPA-A, WGPA-N).	WGPA-A, but not WGPA-N, prevented swim induced enhanced immobility, reduced serum markers of oxidative stress, and protected against ultra-structural changes of striated muscle mitochondria.

(Continued)

TABLE 2 | Continued

References	Study design and sample description	Description	Relevant results
Zhuang et al., 2014	Rats were tested in a model of post-operative fatigue (70% removal of small intestine) using open field activity	Evaluated the antioxidant effect of 15 mg/kg/day Ginsenoside Rb (GRb1) starting 3 days prior to surgery.	On day 1 and 3 post surgery, rats showed reduced activity compared to controls, this reduction was not observed in the GRb1 treated rats. Skeletal muscle SOD, Nrf2, and Akt levels were increased by surgery and further increased by GRb1. Surgery also increased muscle MDA and ROS and these effects were attenuated by GRb1.
METABOLOMIC STUDIES			
CFS			
Yamano et al., 2016	Metabolomics analyses of plasma samples; Samples from patients with CFS ($n = 46$) and healthy controls ($n = 47$); Further validation in a new sample of 20 CFS patients ($n = 20$) and healthy controls ($n = 20$).	Metabolites were identified with capillary electrophoresis time-of-flight mass spectrometry. Out of 144 metabolites identified, 31 with large signal/noise ratio and few missing data were observed in both data sets and thus used for group comparisons.	Initial and validation analyses showed a reduction in the ratio of pyruvate/isotrate and of ornithine/citrulline. Pyruvate was increased and isotrate was decreased in samples from CFS patients, suggesting a reduction in TCA cycle activity, possibly due to a disturbed link between glycolysis and the TCA cycle. Ornithine was higher and citrulline was lower in samples from CFS patients, suggesting a reduction in activity of the urea cycle at entry point of the cycle. Interestingly, these steps take place in the mitochondria, while subsequent steps take place in the cytosol.
Naviaux et al., 2016	Metabolomics analyses of plasma samples from patients with CFS ($n = 45$) and healthy controls (39).	Metabolites were identified with targeted, broad-spectrum, chemometric analysis. Out of 612 metabolites assessed, 420 metabolites that could be identified in all samples and were used for analyses.	The most dominant metabolic disturbances identified in both male and female patients with CFS pertained to sphingolipid pathways, driven by a decrease in plasma sphingolipids and glycosphingolipids. These metabolites also correlated with performance status, but associations with fatigue severity were not reported. *Note, lipids have a broad range of action making interpretation of the findings by Naviaux difficult. It has been suggested that the observed reduced levels could be due to reduced physical activity in CFS patients (Roerink et al., 2017). Physical activity levels were not assessed in the study.*
Fluge et al., 2016	Metabolomics analyses of plasma samples from patients with CFS ($n = 200$) and healthy controls ($n = 102$).	Assessed 20 standard amino acids using gas or liquid chromatography-tandem mass spectrometry.	Amino acids that are converted to acetyl-CoA for entry in the TCA cycle were reduced in CFS samples. In addition, amino acids that are converted to TCA cycle intermediates are also reduced, more dominantly so in females with CFS. *Note, the identified amino acids were not associated with fatigue severity, but with BMI and age.*
ANIMAL MODELS			
Ma et al., 2015	Metabolomics analyses of urine from mice treated or not with salidroside (to alleviate fatigue) subjected to a forced swim test.	Liquid chromatography coupled mass spectrometry was performed to identify an "anti-fatigue" profile.	Several metabolites were upregulated by salidroside, such as: geranyl diphosphate (indirectly regulates lipid synthesis and protein degradation), sebacic acid (a product of fatty acid metabolism), and N-acetylserotonin (antioxidant). Salidroside was associated with a down regulation of metabolites, such as: taurine (sulfur amino acid with many biological functions), sorbitol (involved in glucose metabolism), and sebacic acid (can be oxidized to acetyl-CoA and succinyl-CoA).

various agents with antioxidant properties can reduce fatigue-like behavior (Singh et al., 2002a,b Davis et al., 2009; Fu et al., 2010; Zhuang et al., 2014). For these studies, it should be noted that the models of fatigue pose a significant limitation. As the mechanisms underlying CFS are yet ill-defined, the validity of animal models of CFS are questionable. Further, many of these studies induce and quantify fatigue in the same intervention (e.g., forced swim), which is inherently problematic. Therefore, much work is needed to improve our models systems and validate these findings.

In sum, most evidence for an association between fatigue and mitochondrial functioning comes from CFS, indicating lower levels of antioxidants and possible reductions in mitochondrial ATP production. While lower antioxidant levels were not found in cancer patients (Filler et al., 2016), alterations in mitochondrial gene expression do indicate a role for mitochondrial functioning in fatigue. Results from clinical and pre-clinical studies point toward possible beneficial effects of mitochondria-supporting supplements. However, the majority of observational and intervention studies suffer from severe limitations in either sample size or study design. In addition, the exact causes for reduced antioxidant levels or ATP production have not been studied. While inflammation is a likely cause, it is definitely not the only candidate. Thus, additional preclinical research to identify mechanisms, as well as clinical replications in larger samples using placebo controlled-designs and relevant biomarkers as output, are urgently needed.

Metabolomics

Metabolomic studies allow for a broad assessment of alterations in metabolism (**Table 1**). Three such studies have been reported in CFS, each with different results, but all pointing toward reduced metabolic activity. Yamano et al. showed evidence for decreased activity in the TCA cycle and the urea cycle (Yamano et al., 2016). Naviaux reported downregulated metabolites of two classes of lipids in their CFS group (Naviaux et al., 2016) and Fluge reported reduced concentrations of the amino acids that act as precursors for acetyl-Coenzyme A, one of the primary inputs of the TCA cycle, in CFS patients (Fluge et al., 2016). While Yamano et al. and Naviaux et al. did not report on associations between metabolites and fatigue severity, Fluge et al. reported that there were no associations. Rather, they observed associations with age and body mass index, suggesting that their findings were not specific for fatigue in the CFS patients.

There has been limited work on metabolomics in animal models of fatigue; however, in one study of exercise-induced fatigue, several potential "antifatigue" metabolomic biomarkers were identified (Ma et al., 2015). One was geranyl diphosphate, which can indirectly regulate lipid synthesis and protein degradation. Sebacic acid, a consequence of fatty acid metabolism that can be oxidized into metabolic intermediates for the TCA cycle, was another.

In summary, results from the three metabolomic studies point to alterations in lipid and fatty acid metabolism and decreased TCA activity in relation to fatigue, which resemble metabolic changes during chronic low-grade inflammation (**Figure 3**). As the TCA cycle is needed to create the precursors for the oxidative phosphorylation process, decreased activity would indicate reduced ATP production via reduced oxidative phosphorylation. As lipid metabolism, or lipolysis, generates the most energy per gram of substrate, it is the most efficient manner of energy storage. As noted in **Figure 2**, the catabolism of lipids to usable energy is a slow process and mainly utilized during extended energy need, such as chronic inflammation. Thus, alterations in this metabolic pathway might result in reduced availability of efficient energy sources, creating an increased reliance on carbohydrate-based metabolism. In addition, reduced fatty acid availability would interfere with aerobic energy production, increasing the need for anaerobic glycolytic ATP production.

Increased Energy Expenditure in Persistent Fatigue

Several findings point to behavioral-energy expenditure exceeding energy availability in patients with CFS and CRF. For example, one study reported that about half of a group of patients with CFS had perceived energy expenditure levels that exceeded their perceived energy availability (Jason et al., 2009). In addition, a non-pharmacological intervention aimed at decreasing fatigue in patients with CFS was effective only in those patients in whom perceived energy availability and expenditure were matched (Brown et al., 2011). In CRF, we have recently shown that cancer survivors reporting more-severe fatigue exhibited an increased tendency to exert effort (Lacourt et al., under review). Cancer patients actively undergoing treatment did not show this association, but rather showed a decreased inclination for exerting effort. Similarly, Mortimer et al. (2017) reported significant *positive* associations between fatigue severity and average daily caloric expenditure in breast cancer patients after the fourth cycle of chemotherapy. Thus, there is preliminary evidence suggesting that energy expenditure does not match (perceived) energy availability.

Alterations in Circadian Rhythms and Sleep in Persistent Fatigue

As mentioned above, low-grade inflammation-induced changes in metabolism can be mediated by changes in circadian rhythms and sleep, possibly through changes in melatonin rhythmicity (**Box 2**). Below, we discuss the findings on sleep, circadian rhythm, and melatonin in relation to chronic fatigue (see also **Table 3**).

In CFS, poor sleep and altered melatonin rhythmicity, but not alterations in circadian rhythmicity have been reported. Patients with CFS report more sleep disturbances (Russell et al., 2016) and display abnormal sleep progression (i.e., greater cyclic alternating pattern rate) (Guilleminault et al., 2006). In addition, fatigue severity was associated with patient-reported poor sleep (Milrad et al., 2017), which was in turn associated with minor increases in plasma levels of the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α. While no evidence has been found for altered circadian rhythm in CFS (Hamilos et al., 2001; Rahman et al., 2011), there is evidence for altered melatonin rhythmicity. For example, significantly higher nocturnal salivary melatonin levels were seen in adolescents with

BOX 2 | Melatonin.

Melatonin moderates both sleep and circadian rhythm and an important biomarker for sleep and circadian rhythm. Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone which moderates both sleep and circadian rhythms and is specifically known for its importance in sleep onset. Melatonin concentrations show a circadian pattern with slowly increasing levels during the day, leading to maximal levels at the time of sleep onset, followed by a slow decrease until it reaches a minimum level in the morning when it is time to wake up. In addition to its function for sleep, melatonin can also act as an antioxidant and immune regulator, playing a role in mitochondrial DNA protection (Ramis et al., 2015). Studies in rodents confirm that inflammation and stress are capable of modulating melatonin levels (Persengiev et al., 1991; Huang et al., 2014) and that melatonin can reduce inflammation and oxidative stress associated with sleep deprivation (Kim et al., 2012; Zhang et al., 2013). However, melatonin administration may be insufficient to restore sleep-wake rhythmicity (Mirmiran and Pevet, 1986).

CFS who report unrefreshing sleep compared to healthy subjects (Knook et al., 2000). Further, the association between body temperature circadian rhythm and melatonin onset observed in healthy individuals was absent in patients with CFS (Williams et al., 1996). Although treatment with melatonin or phototherapy (daylight therapy) did not alleviate fatigue in CFS patients in one study (Williams et al., 2002), melatonin treatment was successful in reducing fatigue when participants were selected for a later-than-usual evening-melatonin onset (van Heukelom et al., 2006). The latter study did not include a placebo-control but fatigue reductions were significantly more pronounced in patients with very late onset vs. relatively early onset, suggesting that the effects are not likely explained by placebo effect alone.

In cancer patients, associations between CRF and disrupted circadian rhythm and sleep have been reported before, during, and after cancer treatment (Miaskowski et al., 2011; Payne, 2011; Ancoli-Israel et al., 2014). In a longitudinal study of breast cancer patients, increases in fatigue during chemotherapy were related to increased reports of sleep disturbances as well as increased nap time and decreased wake time during the day (Liu et al., 2012b). In the subset of these patients, for whom information on inflammation was available, increases in either fatigue or disturbed sleep were associated with increased inflammation (Liu et al., 2012a). Interventions aimed at normalizing circadian rhythm have proven successful in alleviating CRF. Bright white light therapy was effective in both preventing and treating circadian-rhythm desynchronization in patients with breast cancer (Neikrug et al., 2012) and improved fatigue in breast cancer survivors (Redd et al., 2014) and in a mixed sample of nonmetastatic cancer survivors (Johnson et al., 2017). Light treatment during chemotherapy for breast cancer was shown to prevent increases in fatigue, although surprisingly, these effects were not mediated by concurrent effects on circadian rhythm (Ancoli-Israel et al., 2012).

In summary, disturbed sleep and altered circadian or melatonin rhythmicity occur frequently among individuals with persistent fatigue, although persistent fatigue can be experienced in absence of sleep and circadian-rhythm alterations. Only a few studies have assessed the associations between low-grade inflammation and sleep or circadian rhythm in the context of fatigue, and the results are inconsistent. In addition, associations between sleep or circadian rhythm and metabolic outcomes have not been studied in fatigued populations.

SUMMARY AND CONCLUSION

We have here proposed a model of persistent fatigue as a consequence of chronic low-grade inflammation leading to an imbalance in energy availability and expenditure, which can be mediated and maintained by changes in circadian rhythms and sleep. The here discussed literature indeed points to associations between persistent fatigue in CFS and CRF with alterations in cellular metabolism, disturbed sleep, and, to a lesser extent, disruptions in circadian rhythm. For both CRF and CFS, multi-causal models are generally suggested (Papadopoulos and Clear, 2011; Moss-Morris et al., 2013; Bower, 2014); not only including low-grade inflammation and disturbed sleep, but also alterations in stress physiological responses, genetic vulnerability, and sociodemographic factors, among others. These causes are not mutually exclusive and feasibly point to a more limited number of final end points. We have here described such a final common pathway, incorporating several recognized contributors to persistent fatigue.

There are several limitations to the current state of knowledge. First, most studies have been carried out in patients with CFS patients, and this is especially the case for studies on metabolism and energy production. It is thus far unclear to what extent findings on metabolism and energy production also pertain to patients with CRF. Further, few reports are available that included more than one of the suggested parameters of our proposed model. Only two studies explored associations between low-grade inflammation and sleep, one in CFS patients and one in fatigued breast cancer patients. To the best of our knowledge, no studies have been published that describe associations between inflammation and metabolism in patients with persistent fatigue. Despite this lack of more-encompassing studies, the evidence thus far suggests that *reduced cellular-energy availability* might play a role in chronic fatigue when the fatigue is associated with *low-grade inflammation*. In addition, there is limited evidence *for increased behavioral-energy expenditure* suggesting that energy expenditure is not adapted to the level of fatigue or of energy availability.

Intervention studies aimed at improving aspects of the model presented, with the ultimate goal to alleviate fatigue, are still scarce. Nevertheless, it is already apparent that participants for such studies should be selected carefully and not solely on the basis of their fatigue experience. This is nicely illustrated by the study by van Heukelom et al. (2006), in which careful selection of individuals with CFS *with* later-than-usual evening-melatonin onset revealed significant beneficial effects of melatonin supplementation. Such effects were not observed when

TABLE 3 | Overview of discussed studies on the association between fatigue and sleep/circadian rhythm.

References	Study design and sample description	Description	Relevant results
OBSERVATIONAL STUDIES			
CFS			
Russell et al., 2016	Prospective diary and actigraphy study; Patients with CFS ($n = 27$) followed for 6 days.	Diaries captured subjective sleep and presleep arousal, mood, and fatigue; Actigrahy data was used to capture sleep efficiency and sleep fragmentation.	Subjective sleep predicted following-day fatigue. Actigraphy-captured sleep quality measures did not predict following-day fatigue. *As no control group was included, it is unclear whether these associations are specific for patients with CFS.*
Guilleminault et al., 2006	Cross-sectional observational study; Patients with CFS but without reported sleepiness ($n = 14$) vs. healthy controls ($n = 14$).	Patient-reported sleep and sleep disruptions; EEG output of one night to capture duration and frequency of sleep cycles and respiratory measures.	Patients with CFS more often reported disrupted sleep. EEG output indicated several subtle differences in the CFS group compared to the controls indicative of abnormal sleep progression and NREM sleep instability. *As patients with CFS also showed increased respiratory effort and nasal flow limitation, the authors argue that abnormalities in sleep progression might be due to underlying undiagnosed apnea.*
Milrad et al., 2017	Cross-sectional study; Patients with CFS ($n = 60$)	Patient-reported sleep and fatigue in association with plasma levels of inflammatory mediators IL-6, TNF-a, and IL-1B.	Greater fatigue severity was associated with worse sleep quality and increased inflammation. Reduced sleep quality was in addition related to increased inflammation. *Associations between fatigue and inflammation were not reported. As no control group was included, it is unclear whether the reported associations were specific for CFS.*
Hamilos et al., 2001	Cross-sectional study; patient with CFS ($n = 10$) vs. controls ($n = 10$)	Circadian rhythm of body temperature assessed in 5-min intervals over 48 h.	Circadian rhythms did not differ between CFS and controls. There was a tendency for greater variability on rhythm in the CFS group. *The authors conclude that disturbance in body temperature circadian rhythm is an unlikely cause of CFS symptoms.*
Rahman et al., 2011	Cross-sectional study; Patients with CFS ($n = 15$) vs. controls ($n = 15$)	Group comparisons on diurnal cortisol concentrations (assessed for one day), circadian rhythm, sleep efficiency and fragmentation (from actigraphy data assessed over 5-days), and self-reported activity and symptoms in 5-day diary assessment.	Patients and controls did not differ in diurnal cortisol patterns or concentrations, circadian rhythm, or objective sleep measures. Patients with CFS reported poorer sleep quality.
Williams et al., 1996	Cross-sectional study; Patients with CFS (n=20) vs. controls ($n = 17$)	Group comparisons of circadian rhythm (24-hr continuous body temperature; patient-reported physical activity levels in 30 min intervals), and dim light melatonin onset (DLMO; time of first rise in melatonin between 18:00 and 24:00).	Groups did not differ in circadian rhythm (of body temperature) and timing of DLMO. DLMO and peak in body temperature (acrophase) were associated in controls, but not in CFS patients.
Knook et al., 2000	Cross-sectional study; Adolescents with CFS ($n = 13$) vs. controls ($n = 15$)	Group comparisons on self-reported sleep onset and duration, sleep quality and sleep problems as well as changes in salivary melatonin concentrations between 17:00 and 02:00.	Adolescents with CFS more often reported unrefreshing sleep, nocturnal wake-ups, and restless sleep. Salivary melatonin level increased during testing in both groups; the increase was stronger in the CFS group resulting in higher levels between midnight and 02:00 a.m.
CRF			
Miaskowski et al., 2011	Cross-sectional study; Cancer patients planned for radiation therapy ($n = 185$; mixed group of breast, prostate, lung, or brain cancer)	Correlations between self-reported sleep disturbances and fatigue as well as objective assessment of sleep quality and circadian rhythm through	Small to moderate associations were found between higher patient-reported fatigue and patient-reported poorer sleep.

(Continued)

TABLE 3 | Continued

References	Study design and sample description	Description	Relevant results
		actigraphy prior to onset of radiation therapy.	Some small correlations were found between fatigue and objective measures of sleep and between fatigue and circadian rhythm (acrophase: the clock time of the peak amplitude was later in patients with higher fatigue).
Liu et al., 2012a,b	Longitudinal observational study; Breast cancer patients scheduled to receive (neo)adjuvant chemotherapy ($n = 97$); data on inflammation was available in a subset ($n = 53$).	Correlations between changes in fatigue, objective/subjective sleep, and inflammation during chemotherapy with assessments made at baseline and during chemotherapy cycle 1 and 4. Objective sleep parameters were obtained from actigraphy data. Associations between fatigue and sleep with inflammation were reported in a second paper (Liu et al., 2012b).	Fatigue increased during chemotherapy and was associated with reported sleep disturbances and some objective markers of sleep. Within-time point associations were moderate for fatigue with subjective sleep measures and mostly absent for objective sleep measures (Liu et al., 2012a) Inflammation increased over time and was associated with increases in both fatigue and poorer reported sleep (Liu et al., 2012b).
CLINICAL TRIALS			
CFS			
Williams et al., 2002	Within-subjects randomized controlled clinical trial; Patients with CFS ($n = 42$)	Effects of melatonin (5 mg/night for 12 weeks) and phototherapy (30 min of bright light therapy/morning for 12 weeks) on body temperature circadian rhythm, melatonin secretion profiles and several patient-reported outcomes. The order of melatonin and phototherapy intervention was randomized and each intervention was preceded by 12 week of placebo.	Neither intervention affected patient-reported symptoms, including fatigue and sleep disturbances. The interventions also did not affect circadian rhythm (with the exception of a slight change in acrophase in the phototherapy intervention) or DLMO. *Of the 42 participants who were randomized, only 30 completed the study.*
van Heukelom et al., 2006	Clinical trial; 29 patients with CFS and late DLMO (>21:30)	Effects of melatonin (5 mg per day, 5 h before DLMO for 3 months) on patient-reported fatigue. Fatigue was assessed before and after treatment.	Melatonin treatment decreased fatigue. This effect was driven by patients with later DLMO (>22:00, n=21), as scores did not improve in those with relatively early DLMO (n=8). *Study lacks placebo-control condition.*
CRF			
Ancoli-Israel et al., 2012; Neikrug et al., 2012	Randomized controlled trial; Breast cancer patients undergoing chemotherapy ($n = 39$) randomized in 2:1 ratio	Effects of 30 min of morning exposure to bright white light (BWL) vs. dim red light (DRL) (placebo) therapy throughout the first 4 cycles of chemotherapy on circadian rhythms (captured with actigraphy over 3 consecutive days) and patient-reported fatigue. Assessments were made at baseline, during treatment in cycle 1 and 4, and during recovery after cycle 1 and 4.	BWL protected against the reductions in activity and rhythmicity as well as increases in fatigue that were observed in the DRL group; changes in fatigue were not mediated by or associated with changes in sleep or circadian rhythms. *Fatigue was increased in the DRL group only during treatment and not in the recovery assessments, while circadian rhythmicity changes were more persistent, suggesting that the observed changes in fatigue were very transient and not reflective of more persistent fatigue that is reported by some breast cancer patients.*
Redd et al., 2014	Randomized controlled trial; Cancer survivors ($n = 36$; mixed diagnoses) up to 3 years post primary cancer treatment randomized in 1:1 ratio	Effects of 30 min of morning exposure to BWL or DRL for 4 weeks on patient-reported fatigue.	BWL lead to consistent improvements in fatigue with lowest fatigue at last assessment, 3 weeks after completion of the intervention end point. DRL led to an improvement in fatigue in week 2 of the intervention, followed by an increase back to baseline. *Effects could not be explained by changes in depression.*
Johnson et al., 2017	Randomized controlled trial; Cancer survivors ($n = 81$; mixed diagnoses) having completed primary cancer treatment randomized in 1:1 ratio	Effects of 30 min of morning exposure to BWL or DRL for 4 weeks on patient-reported fatigue.	BWL led to consistent improvements in fatigue whereas DRL led to some improvement only up to week 2.

participants were selected solely on the basis of their fatigued state (Williams et al., 2002).

In sum, more research is needed to piece together the puzzle explaining how chronic low-grade inflammation can lead to the experience of persistent fatigue. Nevertheless, reduced cellular energy paired with increased or maladaptive changes in energy expenditure poses a potentially important explanation. Evidence for this model can be found for both low-grade inflammation and fatigue.

AUTHOR CONTRIBUTIONS

TL, EV, and CH: contributed to conception of the model proposed in the review; TL: wrote the first draft of the manuscript; EV and GC: wrote sections of the manuscript; RD and CH: reviewed the manuscript at several stages and contributed to fine-tuning of the ideas presented in the review. All authors contributed to manuscript revision, read, and approved the submitted version.

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Age Dependent Hypothalamic and Pituitary Responses to Novel Environment Stress or Lipopolysaccharide in Rats

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Previously, we have shown that the transcription factor nuclear factor interleukin (NF-IL)6 can be used as an activation marker for inflammatory lipopolysaccharide (LPS)-induced and psychological novel environment stress (NES) in the rat brain. Here, we aimed to investigate age dependent changes of hypothalamic and pituitary responses to NES (cage switch) or LPS (100 μg/kg) in 2 and 24 months old rats. Animals were sacrificed at specific time points, blood and brains withdrawn and analyzed using immunohistochemistry, RT-PCR and bioassays. In the old rats, telemetric recording revealed that NES-induced hyperthermia was enhanced and prolonged compared to the young group. Plasma IL-6 levels remained unchanged and hypothalamic IL-6 mRNA expression was increased in the old rats. Interestingly, this response was accompanied by a significant upregulation of corticotropin-releasing hormone mRNA expression only in young rats after NES and overall higher plasma corticosterone levels in all aged animals. Immunohistochemical analysis revealed a significant upregulation of NF-IL6-positive cells in the pituitary after NES or LPS-injection. In another important brain structure implicated in immune-to-brain communication, namely, in the median eminence (ME), NF-IL6-immunoreactivity was increased in aged animals, while the young group showed just minor activation after LPS-stimulation. Interestingly, we found a higher amount of NF-IL6-CD68-positive cells in the posterior pituitary of old rats compared to the young counterparts. Moreover, aging affected the regulation of cytokine interaction in the anterior pituitary lobe. LPS-treatment significantly enhanced the secretion of the cytokines IL-6 and TNFα into supernatants of primary cell cultures of the anterior pituitary. Furthermore, in the young rats, incubation with IL-6 and IL-10 antibodies before LPS-stimulation led to a robust decrease of IL-6 production and an increase of TNFα production by the pituitary cells. In the old rats, this specific cytokine interaction could not be detected. Overall, the present results revealed strong differences in the activation

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patterns and pathways between old and young rats after both stressors. The prolonged hyperthermic and inflammatory response seen in aged animals seems to be linked to dysregulated pituitary cytokine interactions and brain cell activation (NF-IL6) in the hypothalamus-pituitary-adrenal axis.

Keywords: novel environment stress, lipopolysaccharide, fever, hyperthermia, cage switch, NF-IL6, immune-to-brain communication, aging

INTRODUCTION

Stress is an omnipresent stimulus in animals and humans i.e., an alarm reaction as first outlined by Hans Selye in 1936 (Rochette and Vergely, 2017). Evolutionary conserved mechanisms are known to orchestrate the stress response via the autonomic nervous system and the hypothalamus pituitary adrenal (HPA) axis leading to adjustments in effector organs and preparing the "fight and flight" reaction. Interestingly, this stress response also includes an increase in body core temperature, which as well seems to be a common beneficial mode of action to fight the stressor. Indeed, humans and a variety of animal species including baboons, pigs, rabbits, squirrels, rats, mice (Bouwknecht et al., 2007) or wild living impala (Meyer et al., 2008) show such an increase in core body temperature, namely stress-induced hyperthermia. Again, a variety of stressors can elicit this hallmark of stress reaching from so-called stage fright or in German literally translated "lamp fever," when giving a speech in front of a big audience or before examinations in humans (Marazziti et al., 1992), to novel environment, restraint, capture, or social defeat in animals (Oka et al., 2001; Bouwknecht et al., 2007; Meyer et al., 2008; Nakamura, 2015). Interestingly, such an increase in body core temperature can persist for weeks to even years when the psychological stress is exposed repeatedly and/or is of high intensity; a scenario like this is termed "psychogenic fever" (Timmerman et al., 1992; Oka et al., 2001; Nakamura, 2015). In a previous clinical study up to half of cases of high body temperature that were not related to any common abnormalities were diagnosed as psychogenic (Nozu and Uehara, 2005). In rats, for example, social defeat stress induces chronic hyperthermia lasting at least 8 days after cessation of the stressor (Hayashida et al., 2010).

Recently, Nakamura and colleagues have revealed important insights into the neural circuit for psychological stress-induced hyperthermia using experimental animal studies (Nakamura, 2015). For example, the medial prefrontal cortex seems to be one higher brain structure that can activate the dorsal portion of the dorsomedial hypothalamus (DMH), which in turn, directly stimulates sympathetic premotor neurons in the rostral medullary raphe region to increase thermogenesis in brown adipose tissue and to decrease heat dissipation via vasoconstriction (Nakamura, 2015). Thus, this process ultimately leads to an increase in body core temperature i.e., hyperthermia. Moreover, the ventral portion of the DMH drives activation of the paraventricular nucleus (PVN), the first step in HPA axis action. In more detail, corticotropin releasing hormone (CRH) is secreted from parvocellular neurons of the PVN via the median eminence (ME) into the portal circulatory system to activate adrenocorticotropic (ACT) cells in the anterior pituitary lobe. These, in turn release ACT hormone (ACTH) into the circulation, which increases plasma levels of glucocorticoids derived from the adrenal cortex (Papadimitriou and Priftis, 2009).

Previously, we used a model of novel environment stress (NES i.e., cage switch) in rats and have shown that nuclear factor interleukin 6 (NF-IL6), a pivotal transcription factor during inflammation in the central nervous system (Ejarque-Ortiz et al., 2007; Damm et al., 2011; Pulido-Salgado et al., 2015; Schneiders et al., 2015), is also activated in brain structures of the HPA-axis including the anterior lobe of the pituitary (Damm et al., 2011; Fuchs et al., 2013). NF-IL6 not only serves as spatiotemporal activation marker during inflammatory and psychological stress but as well appears to be involved in the modulation of the stress response (Rummel, 2016). Indeed, NF-IL6 was activated in ACT cells and seemed to contribute to tumor necrosis factor (TNF)α expression accompanied by inhibition of ACTH release (Fuchs et al., 2013). However, NF-IL6 deficient mice were capable of mounting a normal HPA-axis response, while the circadian rhythm of circulating glucocorticoid levels was disturbed (Schneiders, 2015).

In the aging population of the western world, changes in the stress and HPA-axes are prone to occur; distinct patterns of diurnal cortisol and a lack of social support and emotional regulation capacities might contribute to a disappearing resilience for coping with psychological stress and infectious diseases in older adults (Gaffey et al., 2016). In a previous study, we revealed that fever, the actively controlled hallmark of systemic lipopolysaccharide (LPS)-induced inflammation, is prolonged in aged rats (Koenig et al., 2014). Moreover, using the cage switch paradigm of NES (unpublished observation), we observed a prolonged hyperthermic response in 24 months aged rats compared to their young 2 months old counterparts, although previous reports revealed no age dependent difference (Foster et al., 1992) or even a smaller response to psychological stress in the aged (Wachulec et al., 1997).

Here, we aimed to further investigate the cage switch-induced hyperthermic response in aged compared to young rats and to reveal more insights into age dependent LPS-induced changes in NF-IL6 activation in components of the HPA-axes, namely, the PVN, the ME and the pituitary. Plasma IL-6 levels were analyzed as potential circulating mediator, which was previously reported to increase with NES (LeMay et al., 1990; Soszynski et al., 1996) and is involved in NF-IL6-activation (Akira et al., 1990; Damm et al., 2011). The mRNA expression of IL-6, NF-IL6 and suppressor of cytokines signaling (SOCS)3 were used as inflammatory markers in the brain. SOCS3 acts as a

negative regulator of IL-6-signaling, and with some limitations, can be used as indirect marker of its action on the brain (Lebel et al., 2000; Rummel, 2016). Previous reports suggested that prostaglandin E2 (PGE2), a crucial terminal mediator in the induction of the febrile response, might as well be involved in cage stress-induced increase in body core temperature (Kluger et al., 1987; Morimoto et al., 1991). Therefore, the mRNA expression of the rate-limiting enzymes of the prostaglandin synthesis pathway, namely, cyclooxygenase 2 (COX2) and microsomal prostaglandin E synthase (mPGES) were analyzed. In order to detect changes in the mechanisms of HPA-axis activation, we investigated CRH and proopiomelanocortin (POMC), two important factors implicated in the formation of ACTH and the final release of corticosterone. To further gain more information about age related changes of the cytokine network in the pituitary we applied cytokine specific antisera in LPS-stimulated primary cell cultures of the anterior pituitary lobe of aged compared to young rats.

MATERIALS AND METHODS

Animals

The study was performed in young (2 months) and old (23–24 months) male Wistar rats (*rattus norvegicus sp*) with a body weight (BW) of 200–250 g (young male), and 748 ± 27,5 g (old male). For measuring core body temperature and motor activity, biotelemetry transmitters (VM-FR TR-3000; Mini-Mitter, Sunriver, OR) were implanted in the abdominal cavity of the rats at least 1 week prior to the experiment. Before surgery, animals were anesthetized using 60 mg/kg ketamine hydrochloride (Albrecht, Aulendorf, Germany) and medetomidin 0.25 mg/kg (CP Pharma Handelsgesellschaft GmbH, Burgdorf, Germany). Meloxicam [5 mg/kg, subcutaneous (s.c.), Boehringer Ingelheim, Ingelheim, Germany] was administered for analgesic treatment pre- and post-surgery. A data acquisition system (Vital View; Mini Mitter) ensured automatic control of data collection and analysis. Body temperature and motor activity were recorded at 5-min intervals. During the total duration of the experiment (3 days before surgery, during the recovery period and for the experimental procedures) rats were housed individually in a temperature- and humidity-controlled climatic chamber (Weiss Umwelttechnik GmbH, Typ 10′US/+5 to +40 DU, Germany) adjusted to 26°C and 50% humidity, with constant access to water and powdered lab chow. Artificial lights were on from 7:00 to 19:00. Animals were accustomed to the handling procedures at least 3 days prior to the experiment. Animal care, breeding and experimental procedures were conducted in accordance with the German animal protection law and the local Ethics committee "Regional Council Giessen" (ethics approval numbers GI 18/2 Nr. 1/2011, GI 18/2 Nr. 51/2008 and V54-19, c20/15c GI18/2).

Treatment and Experimental Protocols

Experiment 1: Age-Dependent Differences in Response to Novel Environment Stress

Acute psychological stress was induced by use of the NES model. Single housed rats were removed from their home cage and quickly placed into a new experimental Plexiglas cage (different to animal facility housing cages and novel for the animals), whereas control animals stayed in their original surroundings. All cages enabled the rats to smell and see their conspecifics in other cages within the climatic chamber. Additional physiological data were gained by recording body temperature and motor activity of the undisturbed animals on the day preceding the experiment, thereby collecting supplementary control data for analysis without sacrificing additional animals. After 90 min, rats were deeply anesthetized with sodium pentobarbital [160 mg/kg, intraperitoneally (i.p.), Narcoren; Merial, Hallbergmos, Germany], blood samples were collected via cardiac puncture and rats were transcardially perfused with 200–300 ml ice-cold 0.9% saline. The time point was chosen according to a previously demonstrated peak of NF-IL6-activation in PVN and the pituitary in young rats (Fuchs et al., 2013). Brains and pituitaries were quickly removed, frozen in powdered dry ice and stored at −55°C until analysis. All experimental procedures were performed between 08:30 and 12:30.

Experiment 2: Age-Dependent Differences in LPS-Induced HPA-Axis Activation

On the day of the experiment, rats were injected i.p. with LPS (100 µg/kg BW; derived from *Escherichia coli*, serotype 0128:B12; Sigma-Aldrich, Deisenhofen, Germany). Control animals were injected with an equivalent volume of sterile pyrogen-free 0.9% PBS (1 ml/kg; Dulbecco's phosphate-buffered saline; PAA, D-Cölbe). All injections were performed between 11:00 and 13:00. After 24 h, rats were perfused and sampling of brains and pituitaries was performed as described above. We previously found that NF-IL6-activation in the pituitary peaked 8 h after LPS-stimulation and returned to baseline at the 24 h time point (Fuchs et al., 2013), while LPS-induced fever was maintained over 24 h in aged but not young rats using the same dose and serotype of LPS (Koenig et al., 2014). Here, we chose this time point to investigate if activation patterns of NF-IL6 in brains structures of the HPA-axis may be prolonged in aged rats compared to the young counterparts.

Tissue Processing for Immunohistochemistry (IHC) and Real-Time Polymerase Chain Reaction (RT-PCR)

For IHC, coronal 20 µm brain and pituitary sections were cut on a cryostat (HM 500, Microm, Walldorf, Germany), thaw-mounted on poly-L-lysine-coated glass slides and stored at −55°C. Sections encompassed hypothalamic brain structures implicated in the HPA-axis including the PVN and the ME, as well as the pituitary anterior lobe (AL), intermediate lobe (IL) and posterior lobe (PL) and were prepared using the stereotaxic rat brain atlas of Paxinos and Watson (1998) as reference (Paxinos and Watson, 1998). For RT-PCR analysis, another 10–15 consecutive frozen 80 µm sections containing the hypothalamus (bregma 0.50 to−3.5 mm) were stapled on glass slides; the hypothalamus was dissected and stored at −55°C for RNA-extraction.

Real-Time PCR

Total RNA from the collected hypothalamic tissue sections was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription of 1 μg of total RNA was performed using 50 U murine leukemia virus (MULV) reverse transcriptase, 50 μM random hexamers and 10 mM dNTP mix in a total reaction volume of 20 μl (Applied Biosystems, Foster City, CA). Following reverse transcription, quantitative real-time PCR was carried out in duplicate using a preoptimized primer/probe mixture (TaqMan Gene Expression Assay) and TaqMan universal PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). For normalization of cDNA quantities between different reactions, house-keeping gene β-actin (catalog No. 4352340E; Applied Biosystems) was measured as a reference, as its stability during aging and inflammation in the brain had been confirmed in previous experiments (Koenig et al., 2014). The relative expression is presented using the 2-(ΔΔCt) method as previously used (Koenig et al., 2014) and described in more detail (Dangarembizi et al., 2018). The sample values represent x-fold differences from a control sample (given as a designated value of 1) within the same experiment. Assay IDs for the analyzed genes are as follows: IL-6 (Rn01410330_m1); COX-2 (Rn00568225_m1); CRH (Rn01462137_m1); POMC (Rn00595020_m1); NF-IL6 (Rn00824635_s1); SOCS3 (Rn00585674_s1).

Immunohistochemistry

Frozen brain and pituitary sections were briefly air-dried at room temperature (RT) for 7 min and then immersion-fixed in 2% paraformaldehyde (Merck, Darmstadt, Germany) diluted in PBS for 10 min at RT. After three consecutive washes in PBS, the sections were incubated at RT for 1 h with a blocking solution consisting of PBS, containing 10% normal donkey serum (NDS; Biozol, Eching, Germany) and 0.1% triton X-100 (Sigma-Aldrich). Double IHC was performed in order to determine NF-IL6 immunoreactivity in specific cell populations. The primary antibody (AB) rabbit anti NF-IL6 polyclonal IgG (1:5000; cat. Sc-150; Santa Cruz Biotechnology, CA, USA) was applied in conjunction with an additional AB to detect specific cell marker proteins. As such, the rabbit anti NF-IL6 polyclonal IgG was either combined with sheep anti von Willebrand (vW) polyclonal IgG (1:3000; cat. SARTW-IG; Affinity Biologicals, Ancaster, Canada) to stain for endothelial cells or with the mouse anti glial fibrillary acidic protein (GFAP) polyclonal IgG (1:2000; cat. MAB3402; Millipore, Billerica, MA, USA) to detect astrocytes or pituicytes. In additional sets of sections, rabbit anti NF-IL6 polyclonal IgG was used together with mouse anti cluster of differentiation (CD) 68 monoclonal IgG (1:500; cat. MCA341R; AbD Serotec; Oxford, UK) to determine potential colocalization of NF-IL6 signals in brain immune cells. Sections were incubated with a combination of these ABs for 20–22 h at 4°C, followed by three consecutive washes in PBS and visualization with Cy3-conjugated anti-rabbit IgG (1:500; cat. 711-165-152; Jackson Immuno Research, West Grove, PA, USA) for NF-IL6 and Alexa-488-conjugated anti-sheep or anti-mouse IgG for cell type markers (1:500; cat. AZA11015 or AZA21202 respectively, MoBiTec GmbH, Göttingen, Germany) after a 2-h incubation at RT. Sections were counterstained with the nuclear 4.6-diamidino-2-phenylindole (DAPI, 1:1000 dilution in PBS) stain (Mobitec GmbH, Göttingen, Germany) to demonstrate nuclear localization of NF-IL6 immunoreactivity (IR). In order to prevent distracting signals from autofluorescent lysosomal storage bodies that accumulate in many tissues during aging, sections from old animals were additionally treated for 5 min with Autofluorescence Eliminator Reagent® (Merck Millipore, Schwalbach, Germany), followed by two consecutive washes in 70% ETOH (Merck, Darmstadt, Germany) diluted in PBS and two final washes in PBS. Finally, all sections were coated with a glycerol/PBS solution (Citifluor, LTD, London, UK), coverslipped (glass cover slips) and stored at 4°C until microscopic analysis was performed. For control experiments, primary ABs were replaced by species-specific antisera to detect any cross reactivity or unspecific binding. Specificity of the signals of all primary ABs has been confirmed in previous experiments (Damm et al., 2011).

Further information with regard to primary ABs: "NF-IL6: Specificity of the NF-IL6 AB was previously confirmed by preabsorption with the respective blocking peptide (sc-150 P, Santa Cruz) and staining is absent in NF-IL6-deficient animals (Ejarque-Ortiz et al., 2007; Schneiders et al., 2015). Staining patterns confirm previous studies using *in situ* hybridization of NF-IL6 mRNA expression in the mouse brain (Nadeau et al., 2005). The AB was raised against the C-terminus of the protein and recognizes the appropriate bands by western blot (Ejarque-Ortiz et al., 2007; Damm et al., 2011).

Von Willebrand factor: The AB is typically applied to stain endothelial cells with a characteristic morphology and distribution and has been previously used in mice, guinea pigs, and rats (Yamamoto et al., 1998; Konsman et al., 2004; Rummel et al., 2005, 2008). Again, patterns visualized by *in situ* hybridization of vW mRNA expression confirm specificity of labeling (Yamamoto et al., 1998).

Glial fibrillary acidic protein: The cytoplasmatic class III intermediate filament GFAP is broadly used to stain astrocytes in several species including the rat [e.g. Debus et al., 1983; Rummel et al., 2005]. A single band is detected at 51 kDa by Western blot in total brain lysates (manufacturer's data sheet).

Cluster of differentiation 68 (CD68/ED1): This monoclonal mouse AB is raised against rat spleen cells and is commonly applied to detect activated microglia (Bauer et al., 1994; Wuchert et al., 2009), perivascular cells (Graeber et al., 1989) and/or phagocytosing macrophages (Bauer et al., 1994) in rat or mouse brain sections as well as primary glial cultures, which show *de novo* synthesis of the CD68 (Bauer et al., 1994; Damoiseaux et al., 1994).

Microscopical Analysis

A conventional light/fluorescent Olympus BX50 microscope (Olympus Optical, Hamburg, Germany) with a black and white Spot Insight camera (Diagnostic Instruments, Visitron systems, Puchheim, Germany) was used for analyzing the sections and taking images. By means of image editing software (MetaMorph 5.05) the individual images were combined into red/green/blue

color figure plates, brightness and contrast were adjusted and the images stored as TIFF files (Adobe Photoshop 5.05). All sections were processed the same way to enable comparison. Semiquantitative or quantitative evaluation of the targeted sections was performed directly for each experiment either by estimates of density or by counting respective signals. For the first method, a five-point scale was used for rating: –(1) no signals, ± (2) single signals in some cases, + (3) low density; ++ (4) moderate density, +++ (5) high density. 2–3 sections per animal and brain or pituitary structure were evaluated and averaged for each animal and subsequently for each group (means of the means). The second method consisted in counting all nuclear NF-IL6 signals and the total number of DAPI-positive cells of the analyzed brain or pituitary structure (3 – 16 sections per animal). After averaging these data for each animal, the percentage of NF-IL6 positive cells out of DAPI-positive cells was calculated for each group.

Primary Cell Culture of the Anterior Lobe of the Pituitary

As previously described (Fuchs et al., 2013), primary cell cultures of the anterior lobe of the rat pituitary were established from young and old male rats. In more detail, previous publications dealing with primary cell cultures of the anterior pituitary lobe (Crack et al., 1997; Carretero et al., 2003; Lee et al., 2008) were used to establish the present protocol. Moreover, cell culture conditions were adjusted to primary neuro-glial cell cultures of the circumventricular organs [e.g., (Ott et al., 2010)]. The present culture contains 5% ACTH immunoreactive cells and S100 immunoreactive folliculostellate cells, as to be expected for a cell culture of the anterior pituitary lobe (Fuchs et al., 2013). For each experiment, the relative cell density was checked to confirm that even when plating the same amount of cells conditions were stable in between sets of experiments. Although these cultures have been extensively used in the past and are well established, a full characterization of all present cell phenotypes is difficult and has not been performed representing also a limitation of the present study. Two to three animals were sacrificed, quickly decapitated with a guillotine for each preparation, and the heads were immersed in ice-cold 0.1 M phosphate-buffered saline, pH 7.4 (PBS; PAA Laboratories GmbH, Coelbe, Germany). The pituitaries were quickly removed under aseptic conditions and transferred in a Petri dish containing ice-cold oxygenated Earle's Balanced Salt Solution (EBSS; Invitrogen, Darmstadt, Germany). Thereafter, the anterior lobe of the pituitary was dissected and placed in a Petri dish with ice-cold, oxygenated Hanks Balanced Salt Solution (HBSS) devoid of Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany) and supplemented with 20 mM HEPES (Sigma-Aldrich), pH 7.4. The tissue was treated for 90 min at 37°C in a solution of 2 mg/ml dispase-1 (Roche Diagnostics, Mannheim, Germany) in oxygenated HBSS with 20 mM HEPES, pH 7.4. After enzymatic treatment, the pituitary fragments were washed once with HBSS containing 1.0 mM EDTA (Sigma-Aldrich) to inactivate the enzyme, followed by three washes with complete medium, composed of Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Darmstadt, Germany) supplemented with 10% Fetal calf serum (FCS, PAA Laboratories GmbH, Coelbe, Germany), penicillin (100 U/ml) streptomycin (0.1 mg/ml) and 4mM L-glutamine (Biochrom AG, Berlin, Germany). By repeated trituration with a 1 ml Eppendorf pipette tip, the tissue was mechanically dissociated in 2 ml complete medium. The cell number was determined using an improved Neubauer C-Chip (NonoEnTek, Seoul, Korea) and, after dilution to ∼250,000 cells per ml, the cells were plated onto prewarmed glass coverslips (MAGV GmbH, Rabenau, Germany) coated with poly-L-lysine (0.1 mg/ml; Biochrom AG, Berlin, Germany), which formed the bottom of a reusable Flexiperm-micro-12 well (6 mm diameter, Greiner Bio-One GmbH, Solingen, Germany) in order to ensure sufficient cell density despite limited absolute cell number. Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% room air at 37°C. The next day, the medium was exchanged to remove cellular debris. In order to prevent potential stimulatory effects of the FCS, the medium was exchanged with serum-free culture medium after 2 days and experimental treatment procedures were carried out the next day. Cells were incubated with LPS (100 μg/ml) or PBS in serum-free culture medium for 6 h. In addition, cells were pretreated with ABs against the cytokines IL-6 or IL-10 (28.5 μl/ml) (NIBSC, Potters Bar, UK) or solvent (serum-free culture medium) for 30 min before LPS or control stimulation. Bulk ion exchange was used to purify sheep IgG from the crude sheep IL-6 and IL-10 antisera and were a gift by GN Luheshi (Douglas Mental Health University Institute, McGill University, Canada). Application as coating ABs for ELISA enabled to check cross reactivity and specificity for these ABs (Rees et al., 1999; Rummel et al., 2006; Pohl et al., 2009; Harden et al., 2014; Koenig et al., 2014). The supernatants were collected and stored at −55°C for cytokine analysis. Cell culture conditions and treatment protocols (6 h time interval and LPS-dosage) were chosen according to the results of prior experiments performed in-house, which have previously shown to induce a robust increase in IL-6 and TNFα in the supernatant (Wuchert et al., 2008; Fuchs et al., 2013).

Measurement of Cytokines and Corticosterone

Cytokine analysis was performed on blood plasma samples and supernatants from pituitary anterior lobe cell cultures. IL-6 and TNFα levels were determined by means of bioassays based on a dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line and on a cytotoxic effect of TNFα on the mouse fibrosarcoma cell line WEHI 164 subclone 13 as previously reported (Damm et al., 2012). The bioassays showed detection limits of 3 IU IL-6/ml and 6 pg TNFα/ml. Corticosterone levels were analyzed in blood plasma samples using a commercial ELISA kit (ELISA; DRG Instruments GmbH, Marburg, Germany; EIA-4164) according to the manufacturer's instructions. The detection range was 1.13 – 415.75 ng corticosterone/ml.

Data Analysis

For analysis of abdominal temperature, delta T (ΔT) was calculated as temperature of each time point subtracted by

the mean temperature from the time interval −120 to 0 min before onset of the novel environment experiment. Abdominal temperatures were analyzed using a three-way repeated measures ANOVA with the between subjects factor age and treatment and the within subjects factor time. Data were divided into 30 min-intervals for analysis and Bonferroni-correction for multiple comparisons was performed, followed—in case of a significant interaction—by a Tukey *post hoc* test (Statistica 12, StatSoft Europe, Hamburg, Germany). Cumulative data on motor activity, relative expression of IL-6, COX2, POMC, CRH, NF-IL6, and SOCS3 in the hypothalamus, blood plasma concentration of cytokines and corticosterone, were analyzed by two-way ANOVA with age (young vs. old) and treatment (stress vs. control) as between-subject factors (Prism 5 software; GraphPad, San Diego, CA). Given a significant interaction, Bonferroni *post hoc* tests were conducted. The cytokine measurements of cell culture supernatants were analyzed separately for each age group by ANOVA followed by Newman-Keuls multiple comparison *post hoc* tests. NF-IL6 positive cells counts were compared separately for each group by parametric *T*-test. *P* values < 0.05 were deemed statistically significant. Data within the small but highly controlled sample size showed moderate homogenous distribution as expected for a biological system of this matter. D'Agostino & Pearson omnibus normality test (omnibus K2, Prism) was applied and revealed that temperature data for each time point were overall normally distributed as well as cumulative motor activity. Homogeneity of variance in primary cell cultures of the anterior pituitary lobe was lower. Due to the rather low n-number D'Agostino & Pearson omnibus normality test was not applicable in other cases. In our hands, the aim of the study was to detect rather large effect sizes. Overall, we believe even with this limitation, our clear and strong effects that became significant are biologically meaningful and supported by the literature. Outlier testing was applied to exclude any data that was outside of an acceptable variance (graphical). All data are presented as mean ± SEM.

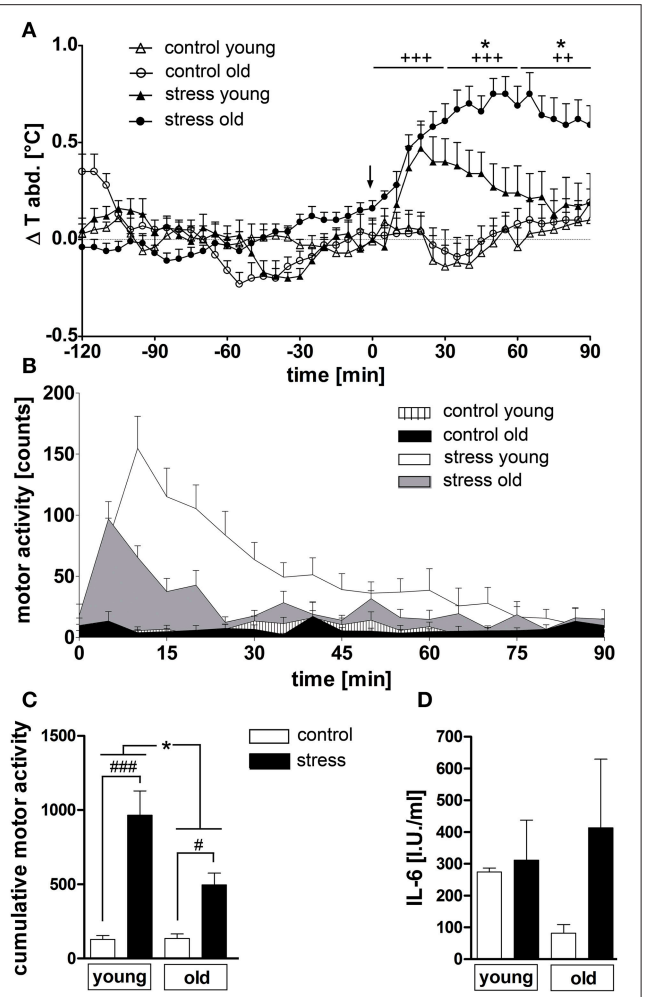

FIGURE 1 | (A) Novel environment stress (beginning of stress indicated by a black arrow) over 90 min induced a rise in abdominal temperature in both age groups, which was strongly enhanced in old male rats compared to young male counterparts. **(B,C)** On the contrary, the stress-induced rise in motor activity, present in both age groups, was significantly higher in the young rats. **(D)** Plasma interleukin 6 (IL-6) levels were not affected by treatment or age. + main effect of treatment (not indicated for **(C)**; * main effect of age; #*post hoc* stress different from control; #, *$P < 0.05$, ++ $P<0.01$, + + +, ###$P <$ 0.001. Control young $n = 12$ (6 in D), stress young $n = 12$ (4 in **D**), control old $n = 13$ (12 in **B,C**, 5 in **D**), stress old $n = 14$ (13 in **B,C**, 5 in **D**).

RESULTS

Influence of Aging on Stress-Induced Hyperthermia and on the Expression of Inflammatory and Stress Mediators

NES induced a rise in abdominal temperature in both age groups (**Figure 1A**). While young rats showed a maximal peak in temperature of 0.47 ± 0.12°C at 20 min, old rats reached a considerably higher peak of 0.75 ± 0.08°C at a later time point of 50 min. Overall, hyperthermia was clearly enhanced in the old rats from 35 to 90 min ($P < 0.05$) compared to the young group and was still present at the end of the experiment in the old animals, while the temperature in the young rats had returned to baseline levels at this time point. Furthermore, NES induced an increase in motor activity in both age groups [main effect of treatment $F_{(1, 45)} = 42.54$, $P < 0.0001$], but this effect was significantly more pronounced in the young rats (*post-hoc* $P < 0.001$) compared to the old group (*post-hoc* $P < 0.05$) [main effect of age $F_{(1, 45)} = 6.372$, $P < 0.05$; age × treatment interaction $F_{(1,45)} = 6{,}725$, $P < 0.05$] (**Figures 1B,C**). Plasma IL-6 levels showed no stress-dependent regulation at this time point, independent of age (**Figure 1D**).

Next, we analyzed a range of relevant inflammatory mediators and markers of HPA-axis activation in order to further investigate the influence of psychological stress on central signaling pathways involved in the manifestation of the febrile response as well as potential age-related differences (**Figure 2**). Analysis of hypothalamic tissue via RT-PCR showed an age-dependent difference in the expression of the inflammatory cytokine IL-6 [main effect of age $F_{(1, 14)} = 13.02$, $P < 0.01$; main effect of treatment $F_{(1, 14)} = 6.833$, $P < 0.05$], revealing that NES led to an upregulated IL-6 expression only in the old rats (**Figure 2A**).

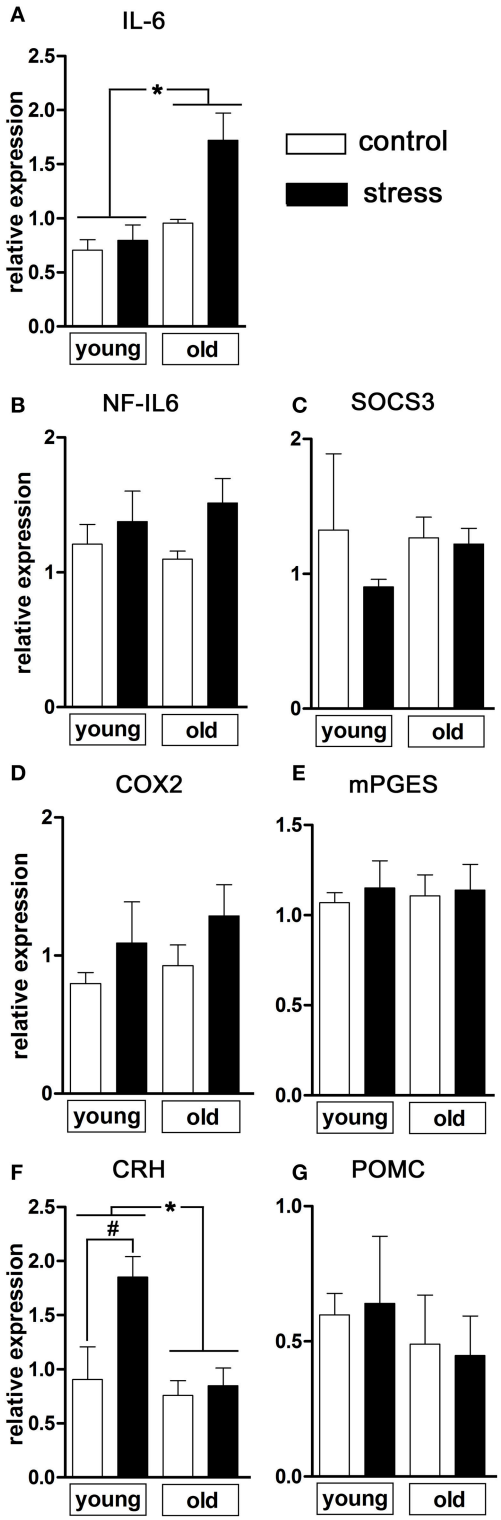

FIGURE 2 | mRNA expression in the hypothalamus of pro-inflammatory mediators and markers of HPA axis activation 90 min after induction of novel environment stress or in unstressed controls in young and old male rats. **(A)** Stress induced an enhanced expression of interleukin (IL)-6 only in the aged rats. **(F)** The expression of CRH (corticotropin releasing hormone), on the *(Continued)*

FIGURE 2 | contrary, was significantly increased after psychological stress only in the young rats while remaining unchanged in the aged group. (**B–E,G**) The other investigated parameters NF-IL6 (nuclear factor interleukin 6), SOCS3 (suppressor of cytokine signaling 3), COX2 (cyclooxygenase-2), mPGES (microsomal prostaglandin E synthase) and POMC (proopiomelanocortin) were not affected by treatment or age. Main effect of treatment not indicated; *main effect of age; $^{\#}$*post-hoc* stress different from control; *, $^{\#}P < 0.05$. Control young $n = 4$, stress young $n = 4$, control old $n = 5$, stress old $n = 5$.

With regard to inflammatory transcription factors, we analyzed the expression of NF-IL6 and of SOCS3 as negative regulator and activation marker for the transcription factor STAT3 (Lebel et al., 2000) (**Figures 2B,C**). In the present experimental paradigm, neither treatment nor aging affected the expression of these factors.

The rate-limiting enzymes of the prostaglandin synthesis pathway COX2 and mPGES remained unaffected by the experimental procedures (**Figures 2D,E**) suggesting that brain derived PGE2 was not involved in stress-induced hyperthermia in our study.

Regulation of hypothalamic CRH expression by psychological stress showed age-dependent differences [main effect of age $F_{(1, 14)} = 8.469$, $P < 0.05$; main effect of treatment $F_{(1, 14)} = 6.905$, $P < 0.05$; age x treatment interaction $F_{(1, 14)} = 4.683$, $P < 0.05$] (**Figure 2F**). While the expression of CRH remained unchanged in old rats (*post-hoc* $P > 0.05$), young rats showed a significant upregulation of CRH expression after NES (*post-hoc* $P < 0.05$). No significant changes were detected in POMC expression (**Figure 2G**).

Additionally to the hypothalamic expression of these two factors, corticosterone plasma levels were analyzed as a complementary terminal readout of HPA-axis activity. Contrary to the previous results for hypothalamic CRH expression, aging upregulated the stress-induced secretion of plasma corticosterone [main effect of age $F_{(1, 17)} = 6.819$, $P < 0.05$] (**Figure 3**).

NF-IL6 IR Is Upregulated in the Pituitary After Psychological Stress in Old Rats

Novel environment stress upregulated NF-IL6 IR in both the anterior and posterior pituitary lobe in old male rats (**Figures 4B,E**). A low basal NF-IL6 activation was already present in unstressed controls (**Figures 4A,D**). These qualitative observations were verified by NF-IL6 cell count (represented as percentage of NF-IL6 positive cells out of all DAPI-positive cells), which clearly confirmed that stressed rats showed significantly increased numbers of NF-IL6 positive cells in the anterior ($P < 0.05$) and posterior ($P < 0.01$) lobes (**Figures 4C,F**). In the ME and the PVN, unstressed control rats showed a high basal activation of NF-IL6 IR (**Figures 4G,J**). Novel environment stress did not lead to any further significant upregulation of NF-IL6 positive cells in the old rats (**Figures 4H,K**), which was also affirmed by cell counts ($P > 0.05$) (**Figures 4I,L**).

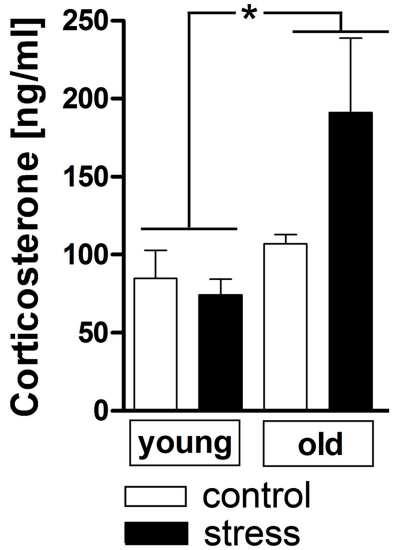

FIGURE 3 | Plasma corticosterone levels after 90 min in stressed and unstressed young and old male rats. Aged rats displayed increased corticosterone levels after psychological stress, while the plasma levels of young rats remained unchanged. *main effect of age; *$P < 0.05$. Control young $n = 7$, stress young $n = 4$, control old $n = 5$, stress old $n = 5$.

Age-Dependent Differences in LPS-Induced NF-IL6 IR and CD68 IR in the Hypothalamus and Pituitary

Based on previous research findings (Fuchs et al., 2013), we aimed to perform a closer investigation of potential age-related differences in LPS-induced activation of NF-IL6 in the brain and HPA-axis. Immunohistochemical analysis of NF-IL6 was performed in the PVN and the ME, because they represent important brain structures implicated in the signal transfer within the HPA-axis. In the PVN, LPS-injection induced only a slight increase in NF-IL6 IR irrespective of age (**Figures 5A–D**). In the ME, a high upregulation of NF-IL6 IR was detected in the old LPS-injected rats (**Figures 5F,H**), while the young group showed only a minor activation (**Figures 5E,G**). An additional semi-quantitative five-point scale evaluation was performed for each group and brain structure and confirmed these qualitative observations (**Table 1**).

Analysis of the complete pituitary structure (anterior, intermediate and posterior lobe; **Figures 6A,B**) revealed that NF-IL6 IR was increased in both age groups after LPS treatment in the posterior lobe and remained unchanged in the intermediate lobe (Supplementary Figure 1). In contrast, inspection of the posterior lobe evidenced an increased NF-IL6 upregulation in the aged group (Supplementary Figure 1B). Closer examination of the posterior lobe revealed that aging did not only increase LPS-induced NF-IL6 IR, but even in the PBS-treated aged control group a higher basal NF-IL6 profile compared to the young counterpart was detected (**Figures 6C–F**). These results were supported by further semi-quantitative evaluation (**Table 2**). Based on these findings, co-localization studies with GFAP and CD68 were performed in order to investigate if increased NF-IL6 activation is connected to a specific cell population, namely pituicytes or CD68 positive immune cells. While no NF-IL-6 activated pituicytes could be detected (Supplementary Figures 2A–D), NF-IL6 signals were seen in CD68 positive cells in all groups (Supplementary Figures 2E-H). Closer analysis of CD68 cells revealed that aged rats showed an increased number of CD68 positive immune cells compared to young rats under basal conditions (**Figures 6G,H**), which was significantly upregulated after LPS-treatment in the aged group (**Figures 6I,J**; see **Table 2** for semi-quantitative analysis).

Aging Affects the Regulation of Cytokine Interaction in the Anterior Pituitary Lobe

LPS treatment significantly enhanced the secretion of the cytokines IL-6 ($P < 0.001$) and TNFα ($P < 0.05$) into supernatants by pituitary cells in the young rats (**Figures 7A,C**), while in the old rats, the increased secretion, although present, was no longer significant for these cytokines (**Figures 7B,D**). Furthermore, in the young rats, the incubation with IL-6 and IL-10 ABs before LPS stimulation led to a robust decrease of IL-6 production ($P < 0.001$ for IL-6 AB and $P < 0.01$ for IL-10 AB) and an increase of TNFα production ($P < 0.05$ for IL-6 AB and $P < 0.001$ for IL-10 AB) by the pituitary cells. In the old rats, this specific cytokine interaction could not be detected.

DISCUSSION

In the present study, we are the first to show that NES-induced hyperthermia is prolonged in aged rats when kept at thermoneutral ambient temperature. This response was accompanied by higher IL-6-expression in the hypothalamus as well as circulating corticosterone in aged rats while plasma IL-6 levels did not show any age dependent changes. CRH mRNA expression was increased by NES only in young but not old rats. Similar to our previous study in young rats (Fuchs et al., 2013), cage switch also enhanced NF-IL6-activation in the pituitary of aged rats. Moreover, nuclear NF-IL6 immunoreactivity slightly or robustly increased in both age groups 24 h after LPS-injection in the PVN and the ME, respectively. Interestingly, ME NF-IL6-activation was significantly higher in the old LPS-stimulated rats than in the young counterparts. In addition, pituitary NF-IL6-activation was higher in LPS-stimulated young and old rats; however, a significant basal activity was only detected in the posterior pituitary lobe of old animals. The higher NF-IL6-activity in both the control and LPS-injected aged groups in the posterior pituitary lobe was partly occurring in and was associated with increased numbers of CD68-positive immune cells.

Earlier studies revealed reduced (Wachulec et al., 1997) or unchanged (Foster et al., 1992) NES-induced hyperthermia in aged compared to young rats. Here, we were able to show prolonged NES induced hyperthermia by cage switch in the aged most likely related to the thermoneutral ambient temperature

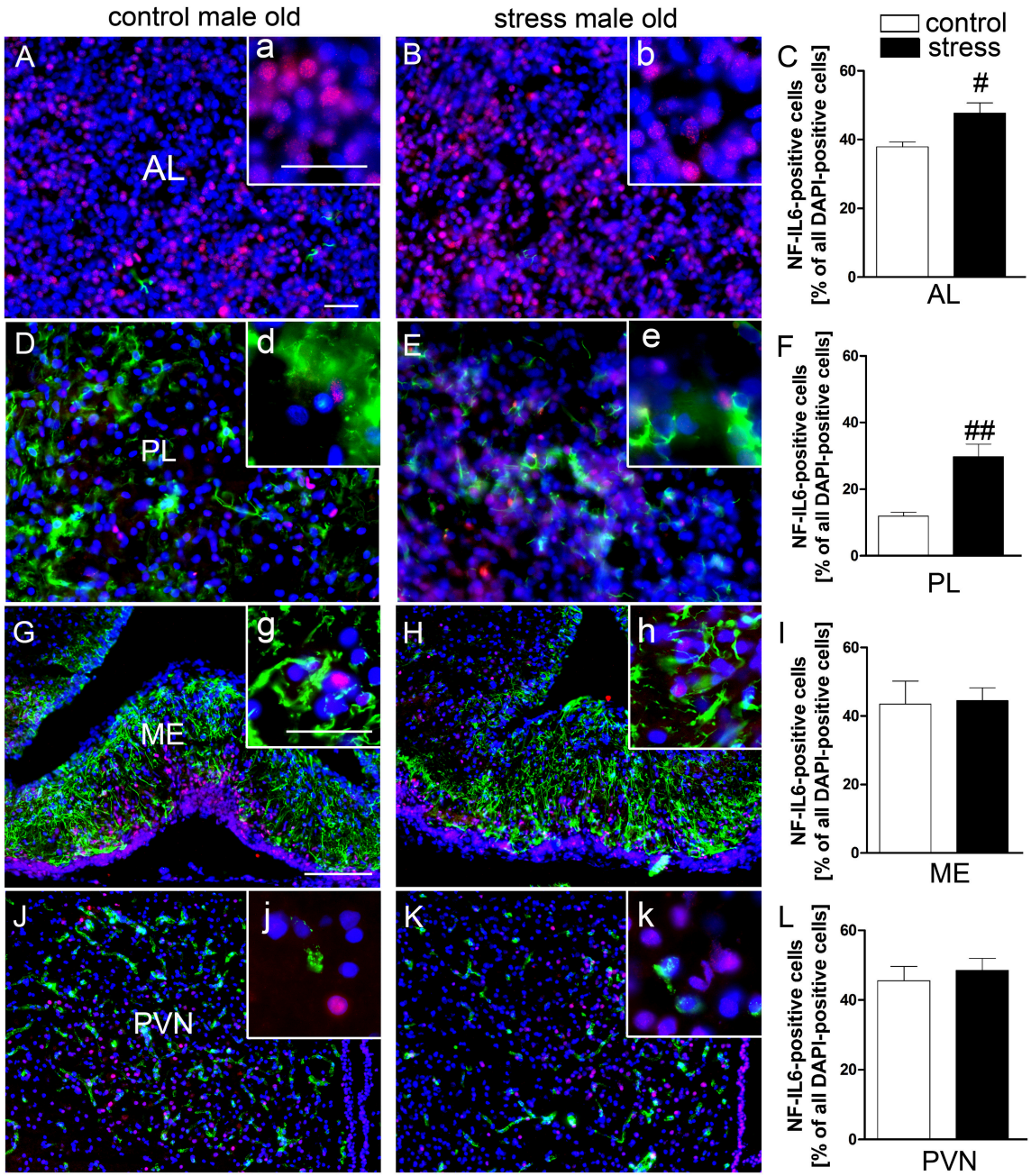

FIGURE 4 | Novel environment stress induced enhanced NF-IL6 (nuclear factor interleukin 6) immunoreactivity (IR, red) in the anterior (AL) and posterior (PL) pituitary lobe **(B,E)** in aged male rats, which was confirmed by further quantitative evaluation (counting of NF-IL6 positive cells) of the immunohistochemistry **(C,F)**. In the median eminence (ME) **(G)** and paraventricular nucleus (PVN) (J) unstimulated controls showed substantial basal NF-IL6 activation. No significant further enhancement of NF-IL6 IR could be observed after novel environment stress **(H,K)** by immunohistochemistry and counting of NF-IL6 positive cells **(I,L)** compared to control animals in these regions. **(A,B,D,E)** GFAP (glial fibrillary acidic protein, green) or **(G,H,J,K)** vW (von Willebrand factor, green) was used to better visualize the pituitary or hypothalamic structures. Cell nuclei were labeled with DAPI (blue). The scale bar in **(A)** represents 25 μm and applies to **(A,B,D,E)**, the scale bar in G represents 100 μm and applies to **(G,H,J,K)**. For all insets (a–k) the scale bar is 25 μm. # stress vs. control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$. Control old $n = 5$, stress old $n = 5$ ($n = 4$ for AL and PL); the mean of 2–3 sections of each animal of the mean for each group was used for quantification.

used in the present study. Indeed, aged animals only show a normal (Florez-Duquet et al., 2001; Buchanan et al., 2003; Peloso et al., 2003) or even prolonged febrile response to LPS (Koenig et al., 2014) when kept significantly above (e.g., 26 or 31°C) the subthermoneutral ambient temperature seen and usually recommended for rodent breeding and housing facilities i.e., around 22°C (Gordon, 2012). In young rats, stress-induced increase in body core temperature does not depend on ambient

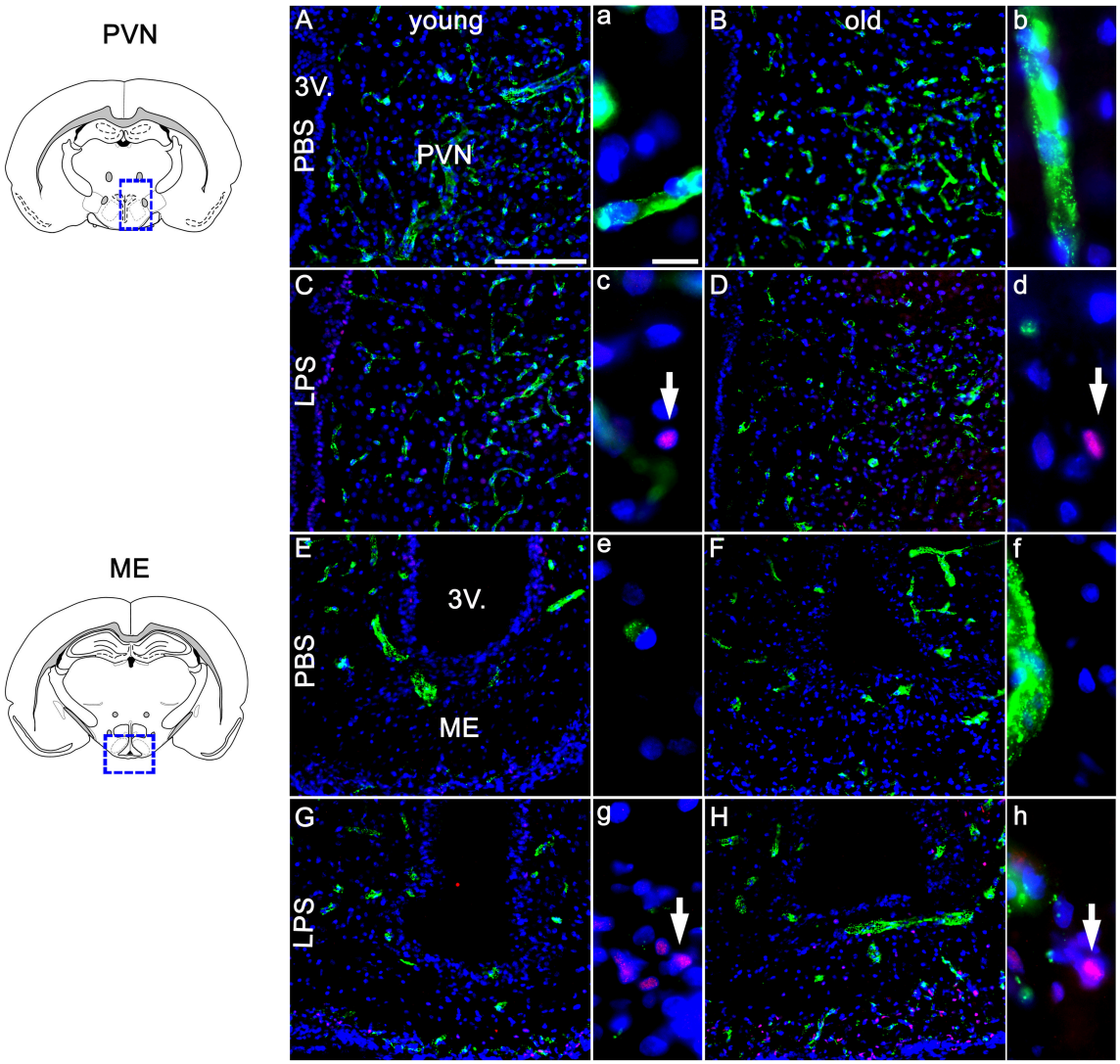

FIGURE 5 | Twenty four hours after LPS-treatment (100 μg/kg), the paraventricular nucleus (PVN, **A–D**) showed scarce activation of NF-IL6 (nuclear factor interleukin 6) signals in isolated cells in young and old rats **(C,D)**, without a clear age-dependent distinction. In the median eminence (ME, **E–H**) however, LPS injection led to a considerably higher expression of NF-IL6 immunoreactivity in the aged group **(H)**, as compared to the young counterpart **(G)**. PBS-injected controls showed no NF-IL6 activation in any group or hypothalamic region. vW (green) was used to better visualize the hypothalamic structures. Cell nuclei were labeled with DAPI (blue). White arrows show nuclear NF-IL6 signals. The scale bar in A represents 100 μm and applies to all overview pictures, the scale bar in a equals 10 μm and applies to all insets. Representative pictures from 3–4 sections per animal; PBS old $n = 5$ for ME, 6 for PVN; LPS old $n = 5$ for ME and PVN; PBS young $n = 5$ for ME, 4 for PVN; LPS young $n = 5$ for ME and PVN.

temperature as already reported by Long et al. (1990a) for 24.7°C and 11.1°C, respectively.

Interestingly, NES-induced increase in body core temperature is not a function of increased locomotor activity. Long et al. (1990b) already found that locomotor activity did not correlate with body core temperature. While some contribution of early and quick increase in body core temperature might be related to increased locomotor activity (Fuchs et al., 2013), clearly, the extent of hyperthermia in particular the prolonged response in the aged rats is not linked to it. Old rats actually display significantly lower locomotor activity than young counterparts. In a previous study, we found altered HPA-axis activity in NF-IL6 deficient animals, which was accompanied by prolonged locomotor activity after NES but no concomitant increase in body core temperature (Schneiders et al., 2015). We hypothesized that CRH might be related to these changes (Schneiders et al., 2015) as intracerebroventricular (i.c.v.) injection of CRH has been shown to increase and its inhibition was demonstrated to reduce locomotor activity (Ohata et al., 2002) and body core temperature in response to cage switch (Morimoto et al., 1993). Indeed, NF-IL6 has been previously shown to activate the CRH promotor (Stephanou et al., 1997). In the present study, we found

TABLE 1 | Semi-quantitative analysis of the amount of nuclear nuclear factor interleukin 6 (NF-IL6) signals in the paraventricular nucleus (PVN) and the median eminence (ME) of young and old male rats 24 h after injection of LPS (100 μg/kg) or PBS.

Brain structure	Nuclear NF-IL6 immunoreactivity			
	PBS young	LPS young	PBS old	LPS old
PVN	− (1,25)	± (2,4)	− (1,17)	± (2,2)
ME	− (1,4)	+ (2,8)	− (1,4)	**++ (4)**

A five-point scale was used to rate the data: − (1) no signals, ± (2) single signals in some cases, + (3) low density; ++ (4) moderate density, +++ (5) high density; n = 3-6 animals per group, 2 sections per animal; the robust difference between young and old is highlighted in bold.

hypothalamic CRH-mRNA expression to be only significantly increased by NES in the young animals while it remained unchanged at basal levels in the aged rats, potentially explaining lower locomotor activity in the aged compared to young animals but not sustained hyperthermia in the old rats exposed to NES.

Overall, these data indicate age-dependent differences between the responsiveness to acute stressors like NES between young and old rats. Possibly, defective stop signals could explain the exaggerated/prolonged hyperthermic response to this acute stressor in the old rats. We found significantly higher corticosterone plasma levels in the aged rats suggesting some resistance related effects for the negative feedback loop of circulating corticosterone on hypothalamic glucocorticoid receptors (Gaffey et al., 2016). It might appear somehow surprising that there was a significant corticosterone increase in plasma of old but not young rats 90 min after NES in the presents study. However, the corticosterone response is rather quick. Previous reports have shown an increase of corticosterone plasma levels in rats already after 10 min open field stress (Morrow et al., 1993) or 60 min after acute pain stress (Rouwette et al., 2011) and ACTH was significantly increased 30 min after cage switch (Morimoto et al., 1991). Thus, the present results after 90 min of NES support the hypothesis of a prolonged corticosterone response in aged rats while corticosetrone already reached basal levels in young animals, which, again might be linked to some resistance to negative feedback mechanisms in the aged. Indeed, for example, suppressive effects of dexamethasone injection on plasma corticosterone were lost in aged rats and to some extent in humans of old age (Wang et al., 1997; Mizoguchi et al., 2009). Such a scenario, however, does not explain the lack of NES-induced CRH-expression in the aged. Therefore, additional mechanisms might contribute to the prolonged NES-hyperthermic response in old rats.

Some previous studies suggest that increased body core temperature in response to cage-switch is rather a fever than hyperthermia. As such, a few studies have shown that plasma IL-6 levels do increase by NES (LeMay et al., 1990; Soszynski et al., 1996) and that the response in the brain is PGE2 dependent using COX inhibitors in an open field stress experiment (Singer et al., 1986; Kluger et al., 1987) or cage switch (Morimoto et al., 1991). Recently, acute 30 min restraint stress as well proved to increase plasma IL-6 and brain COX2 (Serrats et al., 2017). In addition, a glucocorticoid antagonist RU38486 enhanced body core temperature in animals exposed to an open field when injected systemically (Morrow et al., 1993) or i.c.v. (McClellan et al., 1994) again suggesting that old rats may be resistant to glucocorticoid action on thermogenesis during NES. Indeed, we have previously shown that circulating IL-6 induces COX2 (Rummel et al., 2006) and its downstream terminal enzyme mPGES (Rummel et al., 2011) in the hypothalamus and fever using systemic IL-6 injection or an IL-6 antiserum during fever induced by local LPS-induced inflammation. Recently, this observation has been confirmed using genetically modified animals demonstrating an IL-6 activated PGE2-dependent fever induction via brain endothelial cells (Eskilsson et al., 2014). However, the pyrogenic activity of IL-6 is rather weak (Harre et al., 2002; Rummel et al., 2006) and needs to reach a relatively high threshold to activate brain cells and induce fever (Rummel et al., 2004). This threshold can be reduced when IL-6 is combined with low doses of other cytokines such as IL-1 (Cartmell et al., 2000). Interestingly, low circulating IL-6 levels that also occur during exercise seem to correlate with COX2 expression in the hypothalamus (Kruger et al., 2016) but are not accompanied by fever.

The social defeat paradigm and other models of stress-induced increase in body core temperature, which are not PGE2-dependant, clearly differ from fever in underlying brain circuits responsible for vasoconstriction and brown adipose tissue thermogenesis (Nakamura, 2015). While fever is induced via PGE2-stimulated activation of EP3 receptors in the median preoptic nucleus (Lazarus et al., 2007), stress-induced increase in body core temperature in social defeat involves a monosynaptic pathway of glutamatergic activation of the rostral medullar raphe sympathetic premotor neurons (Kataoka et al., 2014), which is not altered by PGE2 synthesis inhibitors (Lkhagvasuren et al., 2011). Similarly, another model of combined stress using i.p. injections followed 1 h later by a novel cage switch did also not reveal any effects of the COX2 inhibitor aspirin on stress-induced rises in body temperature (Vinkers et al., 2009). In the present study, neither plasma IL-6 levels nor COX2- and mPGES-mRNA expression were altered by NES or age supporting that the observed stress-response in this setting represents hyperthermia and not a PGE2-dependent fever. Oka et al. (2001) proposed that PGE2-dependence of psychological stress induced increases in body core temperature might be related to the anticipatory nature of the stress stimulus (Oka et al., 2001).

Apparent discrepancies between the studies that show or do not show effects of COX-inhibitors on the hyperthermic/fever-like responses seem to partially pertain to the intensity of the stress model, e.g., combined or repeated stressors as compared to more moderate stressors such as open filed and cage switch alone. For example, indomethacin was injected 5 h prior to the open field stress by Kluger et al. (1987) as opposed to injection of aspirin 1 h before the NES by Vinkers et al. (2009). In addition, the specific COX-inhibitor type and dose, and modest differences in stress-induced glucocorticoid levels between strains of mice (Romeo et al., 2013) as well as variance in responsiveness to stress between strains of rats might play a role (Porterfield et al., 2011). For instance, it has been shown that Fisher rats are more

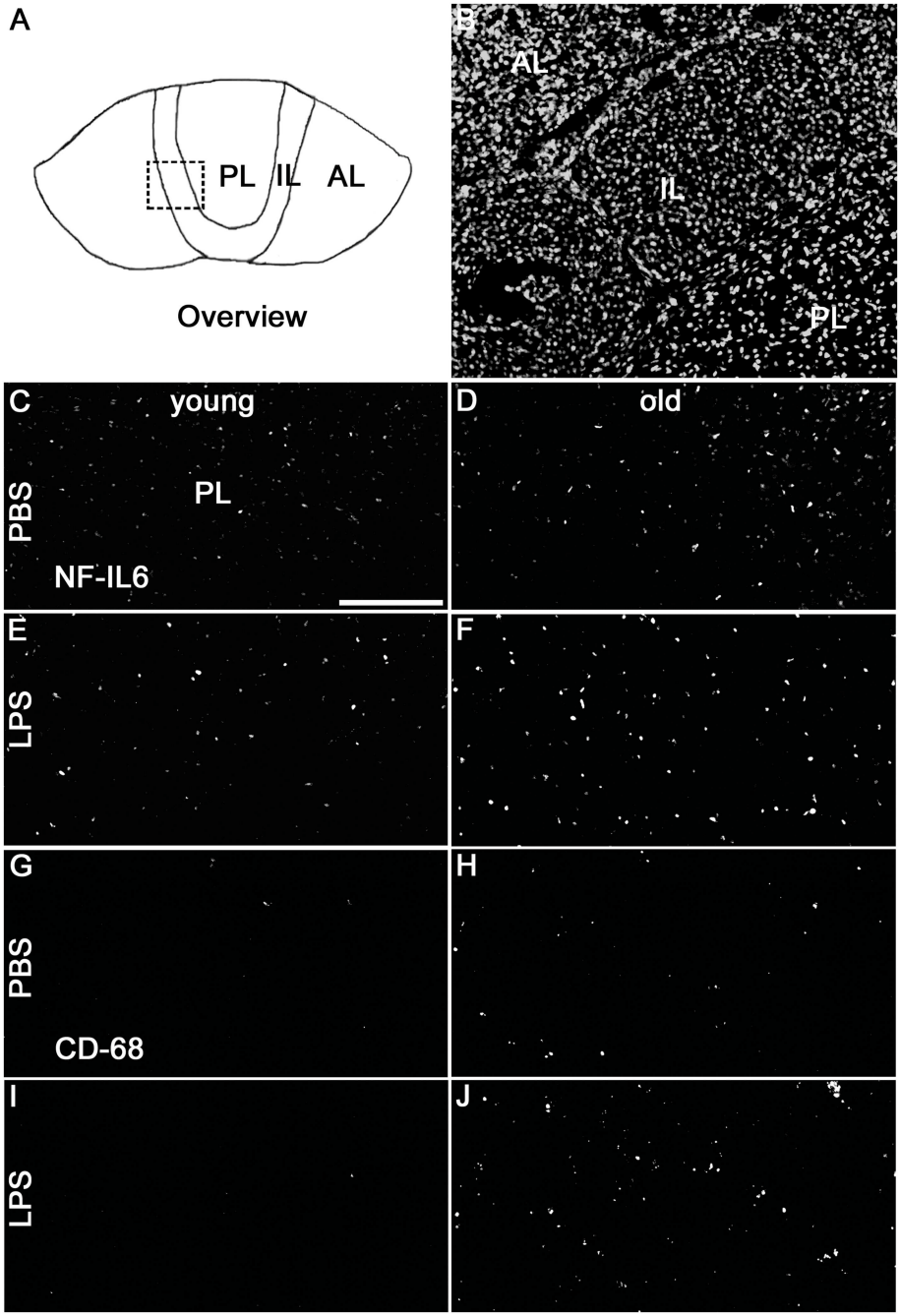

FIGURE 6 | (A) The schematic overview illustrates the structure of the pituitary; the dashed square indicates the substructure represented by DAPI-staining in **(B)**, depicting all pituitary lobes [anterior lobe (AL), intermediate lobe (IL), posterior lobe (PL)]. Old control rats **(D)** displayed a higher basal NF-IL6 (nuclear factor interleukin 6) immunoreactivity (white) compared to young controls **(C)**. After LPS-treatment (100 μg/kg), old rats **(F)** showed highly increased NF-IL6 signals, while young rats **(E)** remained unaffected. CD (cluster of differentiation) 68 immunohistochemistry presented a similar result, with increased numbers in CD68 positive cells (white) in old PBS-injected controls **(H)** and strongly enhanced signals in old rats after LPS-injection **(J)**, while young rats showed only isolated positive cells in both cases **(G,I)**. The scale bar in C represents 100 μm and applies to all overview pictures. Representative pictures from 4-10 sections for NF-IL6 and 2 sections for CD68 per animal; PBS and LPS old $n = 3$, PBS and LPS young $n = 2$.

sensitive than Sprague-Dawley rats to restraint stress-induced IL-1 in the brain (Porterfield et al., 2011) or chronic mild stress-induced plasma glucocorticoid levels (Wu and Wang, 2010) and show higher HPA-axis activity compared to Lewis rat strains (Sternberg et al., 1992; Moncek et al., 2001). Another study demonstrated that some mouse strains like BALB/C mice are

TABLE 2 | Semi-quantitative analysis of the number of nuclear nuclear factor interleukin 6 (NF-IL6) signals and CD (cluster of differentiation) 68 positive cells in the anterior (AL), intermediate (IL) and posterior (PL) lobe of the pituitary in young and old male rats 24 h after injection of LPS (100 μg/kg) or PBS.

Pituitary lobe	PBS young	LPS young	PBS old	LPS old
Nuclear NF-IL6 immunoreactivity				
Anterior lobe	+ (2,7)	++ (3,9)	+ (2,73)	++ (3,87)
Intermediate lobe	± (1,5)	± (1,65)	± (1,67)	± (2,2)
Posterior lobe	± (2,3)	++ (3,73)	**++ (3,5)**	**+ + + (4,87)**
CD68 immunoreactivity				
Anterior lobe	++ (3,5)	++ (4)	++ (3,83)	++ (4,3)
Intermediate lobe	− (1)	− (1)	± (1,83)	± (1,67)
Posterior lobe	± (2,25)	+ (3)	**++ (4)**	**+ + + (4,83)**

A five-point scale was used to rate the data: − (1) no signals, ± (2) single signals in some cases, + (3) low density; ++ (4) moderate density, + + + (5) high density. NF-IL6 immunoreactivity (IR): 2-3 animals per group, 4–10 sections per animal. CD68 IR: 2-3 animals per group, 2 sections per animals; robust differences between young and old are highlighted in bold.

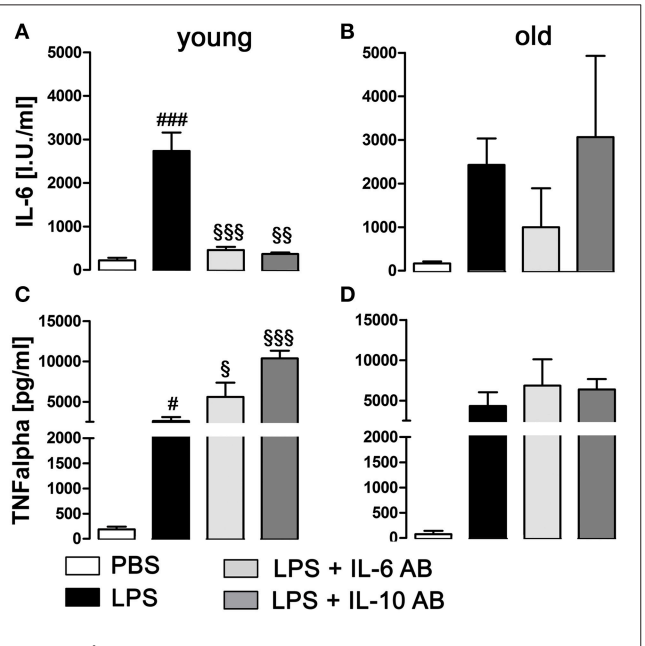

FIGURE 7 | Concentration of tumor necrosis factor alpha (TNFα) and interleukin (IL)-6 in supernatants of primary cell cultures of the anterior pituitary lobe after preincubation with IL-6 or IL-10 antibodies (AB) and/or stimulation with LPS or PBS in young and old male rats. **(A,C)** In young rats, LPS treatment induced a robust increase of IL-6 and TNFα secretion. Preincubation with cytokine ABs decreased the LPS-induced secretion of IL-6 to basal levels, while at the same time increasing TNFα release. **(B,D)** In old rats, there were no significant changes in cytokine secretion, independent of treatment. # LPS vs. PBS; § IL-6 AB/IL10 AB + LPS vs LPS; $^{\#,\S}P < 0.05$, $^{\S\S}P < 0.01$; $^{\#\#\#,\S\S\S}P < 0.001$. Data is derived from at least three independent experiments for each treatment.

more sensitive to CRH antagonist treatment than others (Conti et al., 1994).

As readouts of brain cell activation and inflammation in the brain we used NF-IL6 immunohistochemistry and revealed differences in NF-IL6 activation patterns between young and aged rats. NF-IL6 can be activated by inflammatory (Ejarque-Ortiz et al., 2007; Damm et al., 2011) or psychological stimuli (Fuchs et al., 2013) and regulates the expression of several target genes including IL-6 (Akira et al., 1990). Moreover, NF-IL6-expression and protein levels are increased in the aged brain (Sandhir and Berman, 2010; Koenig et al., 2014). Our present results of increased hypothalamic IL-6-mRNA expression in the aged rats are in line with our earlier results (Rummel et al., 2004) and the inflammatory status, which develops in the brain during aging (Cartmell et al., 2000). Previously, we detected NF-IL6-activation in brain structures of young rats involved in the HPA-axis, namely, the pituitary or the PVN and the pituitary after LPS or cage switch, respectively (Damm et al., 2011; Fuchs et al., 2013) and revealed that NF-IL6 is involved in regulation of basal HPA-axis activity using NF-IL6 deficient mice (Papadimitriou and Priftis, 2009). Now, for the first time, we also found NES-induced activation of the pituitary but not of the PVN in aged rats. Interestingly, the absence of NES-induced NF-IL6 activation in the PVN, which we previously observed in young rats (Fuchs et al., 2013), is in line with the observed lack of increased CRH mRNA expressing after NES in the old rats as this transcription factor can trigger CRH promotor activity (Stephanou et al., 1997).

When using the inflammatory stimulus LPS, we discovered a moderate NF-IL6-activation in the ME, which was significantly stronger in the aged rats 24 h after stimulation. We recently hypothesized a role for ME NF-IL6 activity in potentially gating HPA-axis activity as CRH is expressed in the PVN and released into the ME to trigger ACTH-production and its release from the pituitary (Papadimitriou and Priftis, 2009; Damm et al., 2011). Thus, enhanced LPS-stimulated NF-IL6-activation in the ME of aged rats most likely contributes to dysregulation of the stress-axis during aging. In addition, LPS-induced pituitary activation was more pronounced in the posterior pituitary lobe of aged compared to young rats. Importantly, some of these NF-IL6-positive cells were CD68-positive immune cells but not pituicytes. Moreover, we observed higher numbers of these immune cells in the posterior lobe of the aged rat pituitary potentially signifying some recruitment of activated immune cells. In the brain, CD68 stains activated microglia or perivascular cells (Graeber et al., 1989; Damm et al., 2011). An increase in phagocytosis activity has previously been proposed after LPS-stimulation for the posterior lobe of the pituitary and was also detected by higher CD68 immunoreactivity (Watt and Paden, 2001). However, the enhanced staining of CD68-positive cells in old compared to young rats, which were as well partially NF-IL6-activated, has not been reported before and might represent another mechanism how the brain is targeted by a pro-inflammatory status during aging. Potential immune-cell recruitment is in line with the fact that the posterior pituitary lobe belongs to the circumventricular organs, brain structures with a leaky blood-brain barrier/interface, prone to interact with circulating immune cells and mediators (Roth et al., 2004). As such, perivascular cells contribute to HPA-axis activation during restraint stress as recently revealed by Serrats et al. (2017) using clodronate liposomes to deplete this cell population (Serrats et al., 2017). Thus, these cell types might

as well modulate the function of the posterior pituitary lobe but the exact physiological significance remains to be further investigated. So far, some influence of vasopressin together with CRH for the stimulation of ACTH-release in the anterior pituitary lobe has been suggested and might play some role (Papadimitriou and Priftis, 2009). Otherwise, it is well known that vasopressin regulates blood pressure and water homeostasis in the kidney potentially linked to deficits in water intake and water homeostasis such as high levels of vasopressin and lack of thirst that occur during aging in humans (Begg, 2017). Indeed, water intake is reduced in aged rats using the same dose of LPS (Koenig et al., 2014) and most likely accompanied by high vasopressin levels.

In addition, local cytokine action within the pituitary is also known to contribute to HPA-axis activity regulation and NF-IL6 seems to partially contribute to the regulation of their expression (Fuchs et al., 2013). For example, IL-6 is involved in LPS-induced ACTH secretion via paracrine signaling in the pituitary (Gloddek et al., 2001) while TNFα might act stimulatory (Milenkovic et al., 1989) or inhibitory (Gaillard et al., 1990) on the HPA-axis (Fuchs et al., 2013). Within the so-called cytokine cascade, TNFα can induce IL-6 expression (Van Damme et al., 1987; Zhang et al., 1990). Conversely, IL-6 is known to exert a negatively feedback effect on TNFα production (Schindler et al., 1990) and the anti-inflammatory cytokine IL-10 inhibits expression of both cytokines (Strle et al., 2001). Here, we aimed to investigate potential differences in the paracrine pituitary cytokine network between young and old rats. For this purpose we applied purified antisera against the rat IL-6 and IL-10 on primary LPS-stimulated cell cultures of the anterior pituitary lobe and found for young rats that both cytokine inhibition strategies significantly enhanced LPS-induced TNFα secretion from the anterior pituitary. These results can be explained by afore mentioned inhibitory effects of both cytokines on TNFα production (Schindler et al., 1990; Strle et al., 2001). As expected, IL-6 antiserum significantly reduced IL-6 bioactivity. Interestingly, IL-10 antiserum also reduced IL-6 bioactivity potentially suggesting some overlap of the antiserum on IL-6 in addition to binding to IL-10. Reduced IL-6 bioactivity enhanced LPS-induced TNFα levels with the disappearance of its negative feedback on TNFα. In aged animals, LPS also increased IL-6 and TNF bioactivity in the supernatants but neither IL-6 nor IL-10 antiserum significantly altered the cytokine levels. Overall, these results suggest some deficits that seem to evolve in aged anterior pituitary cells for the cytokine network; i.e., IL-6 levels could not significantly be inhibited by the antisera. Subsequently, TNFα levels were not affected. Thus, local paracrine action of cytokines in the aged pituitary might not be sufficient to adjust appropriate function and might contribute to the overall differences of HPA-axis activity between young and old.

Clinical Perspective

Mixed results have been revealed concerning the impact of aging on the HPA-axis activity in humans (Gaffey et al., 2016). Gaffey et al. (2016) overall conclude that the diurnal rhythm is maintained during aging while cortisol output increases with age. Such a scenario is most likely related to a loss or weakening of the negative feedback loop via glucocorticoid receptors in the brain. Thus, acute challenges result in higher plasma cortisol levels in the old age (Otte et al., 2005), similar to what we observed in the present study in rodents to NES-stress. However, in humans, longitudinal studies will be necessary to get more insights into the altered function of HPA-axis in relation to aging, stress/individual health status and risk to develop disease (Gaffey et al., 2016).

Human diversity in stress response and resilience might show similarities to described species and strain dependant differences as discussed in detail above. A recent study by Rimmerman et al. (2017) using fractalkine deficient mice suggests a role for a differential transcriptome due to fractalkine in hippocampal microglia to convey stress resilience (Rimmerman et al., 2017). Future studies will help to reveal if such mechanisms might also be functional and important for stress resilience in humans.

Overall, rodent models are important to reveal insights into species conserved and species specific underlying mechanisms in evolutionary well conserved responses like stress including stress-induced hyperthermia. In addition to previous advances in brain intrinsic neuronal circuits that explain thermogenesis to psychological stress (Nakamura, 2015), we have added to the current understanding on how aging might affect hypothalamic and pituitary activation patterns and cytokine networks. "Psychogenic fever" is known to occur in humans (Nakamura, 2015) and psychological stress seems to contribute to such processes as for example reported in a recent case report (Oka et al., 2013). Further studies will be necessary in humans and animals to gain more insights into underlying mechanisms that contribute to alteration in the HPA-axis and related fields such as loss of thirst and so-called "psychogenic fever" in old age.

DATA AVAILABILITY STATEMENT

Datasets are available on request.

AUTHOR CONTRIBUTIONS

SK, JR, and CR contributed to the conception and design of the study. *In vivo* experiments and sample preparation were conducted by SK, JB, and CR. Cell culture experiments were performed by SK and FF. SK performed statistical analyses. Data analyses and interpretation were done by SK, JB, and CR. SK, JB, AP, JR, and CR contributed to writing the article and to revising the content. All authors proofread the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00055/full#supplementary-material

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Elevated Plasma Chemokines for Eosinophils in Neuromyelitis Optica Spectrum Disorders during Remission

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Background: A prominent pathological feature of neuromyelitis optica spectrum disorders (NMOSD) is markedly greater eosinophilic infiltration than that seen in other demyelinating diseases, like multiple sclerosis (MS). Eosinophils express the chemokine receptor CCR3, which is activated by eotaxins (CCL11/eotaxin-1, CCL24/eotaxin-2, CCL26/eotaxin-3) and CCL13 [monocyte chemoattractant protein (MCP)-4]. Moreover, CCL13 is part of the chemokine set that activates CCR2. The present study aimed to evaluate plasma levels of eotaxins (CCL11, CCL24, and CCL26) and MCPs (CCL13, CCL2, CCL8, and CCL7) in patients with NMOSD during remission.

Methods: Healthy controls (HC; $n = 30$) and patients with MS ($n = 47$) and NMOSD ($n = 58$) in remission were consecutively enrolled in this study between January 2016 and August 2017. Plasma CCL11, CCL24, CCL26, CCL2, CCL8, CCL7, CCL13, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β levels were detected using the human cytokine multiplex assay.

Results: Plasma CCL13, CCL11, and CCL26 levels were all significantly higher in patients with NMOSD than in HC and patients with MS. No significant differences were found in the CCL13, CCL11, or CCL26 levels between patients with NMOSD receiving and not receiving immunosuppressive therapy. The plasma levels of TNF-α and IL-1β, which stimulate the above chemokines, were higher in patients with NMOSD than in HC. There was no difference in CCL24 levels among the three groups. In most cases, the CCL7 levels were below the threshold value of the human cytokine multiplex assay, which is in line with other studies. Adjusted multiple regression analyses showed a positive association of CCL13 levels with the number of relapses after controlling gender, age, body mass index, and disease duration in patients with NMOSD.

Conclusion: The study indicates that in NMOSD, the overproduction of cytokines such as IL-1β and TNF-α during remission stimulates eosinophilic chemoattractants such as CCL13, CCL11, and CCL26, which in turn bind to their receptor (CCR3); this could lead to eosinophil hypersensitivity. These findings suggest that the elevated secretion of CCL13, CCL11, and CCL26 may be a critical step in eosinophil recruitment during NMOSD remission.

Keywords: neuromyelitis optica spectrum disorders, CCL13, relapse, eotaxin, eosinophil, interleukin-1beta, tumor necrosis factor-alpha

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INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD), an idiopathic inflammatory demyelinating disorder of the central nervous system that involves both the optica nerves and the spinal cord, was considered a subtype of multiple sclerosis (MS) for many years until the discovery of its specific serum antibody biomarker—aquaporin (AQP) 4 IgG (1–3). Although B cells, AQP-4 antibody-dependent complement-dependent cellular cytotoxicity (CDCC), and antibody-mediated cellular cytotoxicity (ADCC) play decisive roles in the pathogenesis of NMOSD, eosinophils have been implicated as an important player in the pathological lesions following blood–brain barrier (BBB) injury (4–6). The basic histopathological features of NMOSD have been described as acute spinal cord lesions with diffuse swelling and softening over multiple segments involving the entire spinal cord or having continuous distribution (7). Autopsy examination has shown that a prominent feature of human NMOSD lesions is markedly greater eosinophilic infiltration than that seen with other demyelinating diseases like MS (8, 9). Eosinophils are also found in the cerebrospinal fluid (CSF) of patients with NMOSD, and demyelinating NMOSD lesions show marked eosinophil infiltration in patients with NMOSD and mouse models (4, 10–12). Lesion severity can increase because of transgenic hypereosinophilia and *vice versa* (4). An interesting finding is the presence of eosinophils in inflammatory demyelinating lesions in NMOSD but their absence in MS. Further, eosinophils have been found in all early active NMOSD lesions but not in cases of acute disseminated encephalomyelitis or acute spinal cord infarction (8). Other studies have proved that the lesion infiltrates are associated with C–C motif chemokine receptor (CCR)-3 expression and stain positively for major basic protein (MBP) in eosinophils, suggesting basic protein release from granules in patients with NMOSD.

CCR3 is highly expressed on the surface of eosinophils. Interactions of CCR3 with a variety of chemokines including CCL11 (eotaxin-1), CCL24 (eotaxin-2), CCL26 (eotaxin-3), CCL13 (monocyte chemoattractant protein 4, MCP-4), CCL8 (MCP-2), and CCL7 (MCP-3) play an important role in the migration, accumulation, and activation of eosinophils (13–15). The "eotaxins," CCL11, CCL24, and CCL26, represent selective chemoattractants for eosinophils (14). Together with CCL11, CCL13 is one of the most important eosinophilic chemoattractants (16). This protein homeostatically recruits eosinophils to organs in disease states. In humans, four MCPs have been identified: MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), and MCP-4 (CCL13) (17). Unlike other MCPs, CCL13 binds not only with CCR1 and CCR2 but also with CCR3, to act as a chemoattractant for eosinophils (18, 19). Several lines of evidence have shown that CCL13 plays a pivotal role in inflammatory cell recruitment in allergic and autoimmune disorders such as rheumatoid arthritis, asthma, parasitic infection, and atopic dermatitis, and it also works in overweight subjects (20–24).

The present study aimed to investigate the factors that induce the effects of eosinophils in NMOSD during remission. Thirty healthy controls (HC), 47 patients with MS, and 58 patients with NMOSD were enrolled to screen cytokines and chemokines. The study showed that plasma levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, CCL13, CCL11, and CCL26 were increased in patients with NMOSD. Additional analysis led to the finding that CCL13 levels were strongly correlated with recurrence times, and we believe that it can be considered one of the most valuable prognostic factors in NMOSD.

SUBJECTS AND METHODS

Subjects

Ethics approval for this study was obtained from the ethics committee of the Beijing Tiantan Hospital Affiliated to the Capital Medical University in China (No. KY2015-003-02). Prior to participation, all patients and HC provided written informed consent.

Patients with NMOSD and MS were recruited from the Beijing Tiantan Hospital between January 2016 and August 2017. The diagnosis of these conditions was confirmed according to the 2015 revised international criteria (25) and the 2010 McDonald's diagnostic criteria (26), respectively. All patients were in remission (had remained relapse-free for over a 1-month period) and were not coexisting any other autoimmune comorbidities at the time of blood collection. Kutzke Expand Disability Status Scale (EDSS) scores were determined from an MS cohort study. Age- and sex-matched volunteers without immune disorders were recruited as HC. Infections were ruled out on the basis of complete blood count testing in all subjects.

Assay for Plasma Chemokines and Cytokine Levels

Peripheral blood was obtained from each subject. To exclude the effect of different time points and other factors on the level of chemokines and cytokines, all blood samples were collected at 9:00 a.m. After collection into a 4-ml disposable BD Vacutainer® containing ethylene diamine tetraacetic acid, plasma samples were separated at 2,000 × *g* for 10 min within 3 h and stored in aliquots at −80°C until further analysis. All testing was performed in a blinded manner to the diagnosis or clinical presentations.

Plasma CCL11, CCL24, and CCL26; CCL2, CCL8, CCL7, and CCL13; and TNF-α and IL-1β levels were assayed using MILLIPLEX® map human High Sensitivity Cytokine/Chemokine Panels (Cat. Nos. HCYTOMAG-60K and HCYP2MAG-62K) (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was conducted using SPSS 22.0 (International Business Machines Corporation, Chicago, IL, USA). After the test of normality, data with non-Gaussian distributions were analyzed using the Mann–Whitney *U*-test for two groups and the Kruskal–Wallis *H*-test for multiple groups by using Dunn's *post hoc* analysis. Normally distributed data were processed using Student's *t* test or analysis of variance. CCL13, CCL11, and CCL26 values of NMOSD patients were used as independent variables in multiple linear regression analysis, with relapse times, annual relapse rate (ARR), and EDSS scores as continuous outcome measure. Gender, age, body mass index (BMI), and duration of NMOSD were included to determine the variables independently

associated with these outcomes. Correlations between TNF-α, IL-1β, and CCL13, CCL11, and CCL26 were assessed using nonparametric Spearman's rank test. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Clinical Demographics

We identified 58 patients with NMOSD, 47 patients with MS, and 30 HC, and their demographic and clinical characteristics are described in **Table 1**. The disease duration and duration to the last relapse in the patients with NMOSD were not significantly longer than that in patients with MS. A significant difference in EDSS scores was found between the NMOSD and MS groups ($P < 0.01$), which was consistent with our previous findings (27).

Plasma MCPs (CCL2, CCL8, CCL7, and CCL13) and Eotaxins (CCL11, CCL24, and CCL26) Levels in NMOSD

Plasma CCL13 levels were significantly higher in patients with NMOSD than in HC and patients with MS. The levels in patients with MS were not significantly higher than in HC (**Figure 1A**). We also examined the levels of other MCPs in all subjects. Unlike CCL13, no significant differences were found in the levels of CCL2 and CCL8 among patients with NMOSD, patients with MS, and HC (**Figures 1B,C**). Further, the CCL7 levels in almost all subjects were below measurable levels (**Figure 1D**).

Plasma CCL11 levels in patients with NMOSD during remission were significantly higher than those in patients with MS and HC, and CCL26 levels showed similar findings. Patients with MS had higher CCL11 levels than the HCs did, but no significant differences were found in CCL26 levels between them (**Figures 2A,C**). No significant differences were found in plasma CCL24 levels among the three groups (**Figure 2B**). Similar results were found when removing the NMOSD outliers in CCL13, CCL11, and CCL26 concentrations and data were shown in Figure S3 in Supplementary Material.

TABLE 1 | Patient demographic and clinical characteristics.

Index	HC	MS	NMOSD
No. of patients	30	47	58
Gender (female/male)	22/8	34/13	54/4
Age (years, mean ± SE)	33.90 ± 1.64	34.96 ± 1.59	39.53 ± 1.57
Age at onset (years, median, and range)	–	30, 6–60	35, 14–62
BMI (kg/m^2, mean ± SE)	21.58 ± 0.44	22.36 ± 0.43	22.80 ± 0.42
Duration of disease (months, mean, and range)	–	40.36, 1–143	56.11, 2–260
Duration to the last relapse (months, mean, and range)	–	5.79, 1–57	5.91, 1–33
No. of relapses (mean ± SE)	–	2.85 ± 0.26	3.35 ± 0.23
ARR (mean ± SE)	–	2.17 ± 0.37	1.62 ± 0.20
EDSS	–	2.64 ± 0.20	3.54 ± 0.21**

HC, healthy controls; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorders; No., number, BMI, body mass index; EDSS, expanded disability status scale; ARR, annual relapse rate.
***P < 0.01.*

Effect of Immunosuppressive Therapy on Plasma Levels of CCL13, CCL11, and CCL26 in NMOSD

Eighty-three percent of the recruited patients with NMOSD were undergoing immunosuppressive therapy [$n = 48$; 27 with oral glucocorticoids and 21 with oral treatment with mycophenolate mofetil, azathioprine, tocilizumab, or cyclosporine A (14 patients received one of these drugs in combination with glucocorticoids)]. Therefore, it was necessary to study whether immunosuppressive therapy affected plasma CCL13, CCL11, and CCL26 levels. The plasma CCL13, CCL11, and CCL26 levels in patients with and without immunosuppressive therapy were both significantly higher than those in the HC, and the levels in patients undergoing immunosuppressive therapy were not significantly lower than those in the other 10 patients (Figure S1 in Supplementary Material). These findings indicated that immunosuppressive therapy had little effect on the three eosinophilic chemoattractant levels.

Regression Analysis of CCL13, CCL11, and CCL26 with Relapse Times, ARR, and EDSS Scores in NMOSD

In NMOSD patients, multiple regression analysis of CCL13, CCL11, and CCL26 with relapse times, ARR, and EDSS scores showed that only CCL13 was significantly related to relapse times ($R^2 = 0.553$, $P = 0.000$) after controlling gender, age, BMI, and disease duration (**Table 2**). Age and disease duration were identified as independent factors associated with relapse times ($P = 0.029$ and 0.024, respectively). Gender and BMI showed no independent and significant regression coefficient with relapse times ($P = 0.158$ and 0.355, respectively). No associations of CCL11, CCL13, and CCL26 with ARR or EDSS scores were found. These data suggested that plasma CCL13 levels may have a prognostic value in NMOSD. Further studies with a larger number of subjects would provide adequate power to detect the differential correlation pattern between CCL13 levels and the clinical characteristics of NMOSD. Similar results were found after removing the NMOSD outliers in CCL13, CCL11, and CCL26 concentrations (Table 1 in Supplementary Material).

Plasma TNF-α and IL-1β Levels and Their Correlation with CCL13, CCL11, and CCL26 Levels

In order to explore the mechanisms underlying the increased CCL13, CCL11, and CCL26 levels in NMOSD, we examined the levels of plasma TNF-α and IL-1β, which have been proposed to stimulate their production. The data showed that both plasma TNF-α and IL-1β levels in patients with NMOSD were significantly higher than those in HC. Further, the TNF-α levels were lower in patients with MS than in those with NMOSD (**Figures 3A,B**). Plasma TNF-α and IL-1β levels were positively correlated with plasma CCL13, and TNF-α levels were also positively correlated with plasma CCL11 levels (Figure S2 in Supplementary Material). No correlation was found between IL-1β and CCL11 ($P = 0.299$),

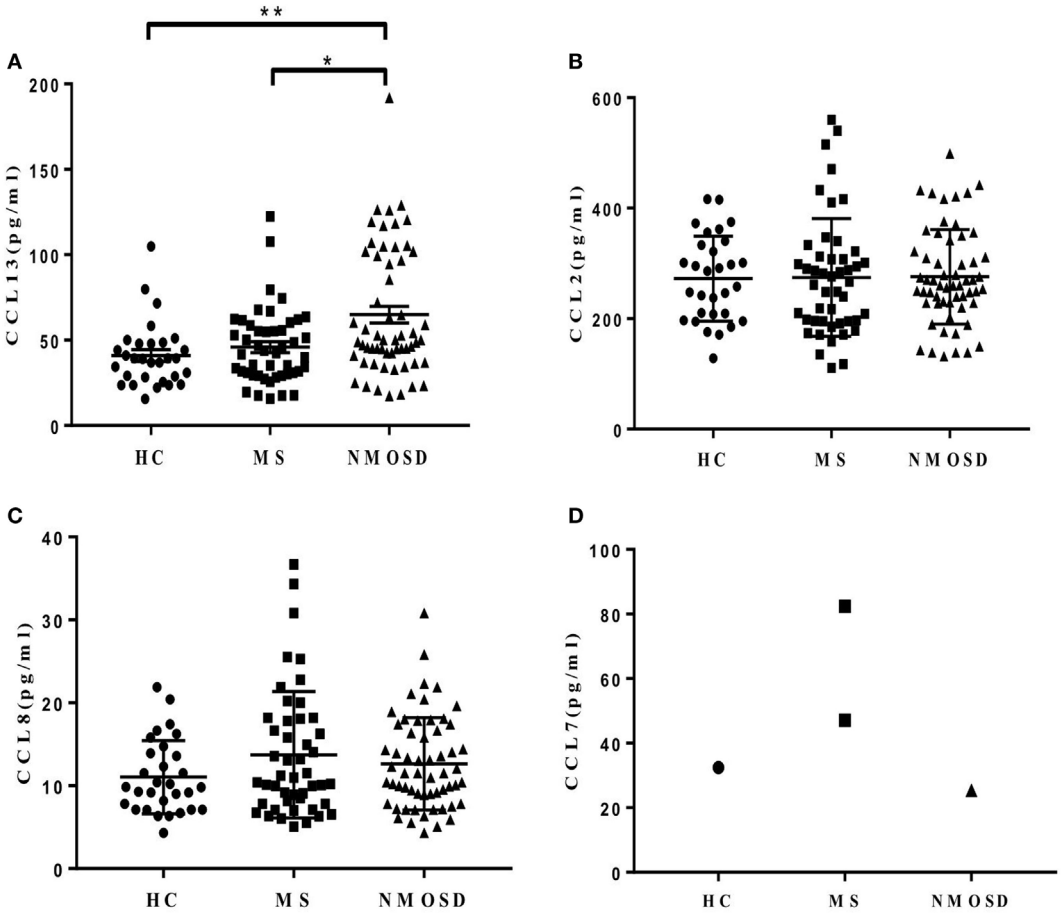

FIGURE 1 | Plasma levels of monocyte chemoattractant proteins (CCL13, CCL2, CCL8, and CCL7). **(A)** Plasma CCL13 levels in healthy control (HC), multiple sclerosis (MS) patients, and neuromyelitis optica spectrum disorder (NMOSD) patients (mean ± SE). **(B,C)** No significant differences of CCL2 and CCL8 levels were found among plasma in HC, MS patients, and NMOSD patients (mean ± SE) ($P = 0.89$ and 0.39, respectively). **(D)** Plasma CCL7 levels in almost all subjects were below measurable levels. Kruskal–Wallis *H*-test and Dunn's *post hoc* analysis were used. $^{*}P < 0.05$, $^{**}P < 0.01$.

and neither TNF-α nor IL-1β levels showed correlations with CCL26 ($P = 0.108$ and 0.297, respectively).

DISCUSSION

Recently available data suggest that low-grade inflammation plays a pivotal role in the development of NMOSD, and low-grade inflammation is frequently observed in patients with NMOSD. As a source of versatile proinflammatory mediators, eosinophils have the unique characteristic-specific granules, which contain four major granule proteins (MBP, eosinophil cationic protein, eosinophil peroxidase, and eosinophil neurotoxin) and numerous cytokines, chemokines, and growth factors (28). These granule releases are the involved infiltrates of eosinophilic ADCC and CDCC response. Histopathologic studies had demonstrated that marked perivascular and meningeal eosinophil infiltrations were showed in active NMOSD lesions and were associated with CCR3 expression (8). CCR3 is a member of the seven transmembrane domain G protein-coupled receptor family, and its major ligands—CCL13 and eotaxins (CCL11, CCL24, and CCL26)—are involved in eosinophil chemoattraction (29).

Similar to other MCPs, CCL13 binds to CCR1 and CCR2 and acts as a chemoattractant for monocytes, T cells, and dendritic cells (DCs), and it is the only MCP that binds to CCR3. Together with CCL11, CCL13 is one of the most important chemoattractants for eosinophils (16). Considering the roles that eosinophils play in NMOSD pathogenesis, CCL13 may act as a distinctive chemoattractant in NMOSD. Compared to other MCPs, CCL13 plays an interesting role in DC migration into inflamed lesions, because monocytes and blood DC precursors such as CD34+-derived DCs and CD11c + DCs respond first to CCR2 ligands and CCL13 in epithelial cells present in contracted blood vessels, and these cells could then be recruited into inflamed tissue by a different gradient (30, 31). In addition, one subtype of Th17 cells that could induce the production of the AQP-4 antibody is distinguishable from its expression of CCR2 + CCR5− and RORγt, and ligands of CCR2 like CCL13 may be important regulators in the recruitment of Th17 cells in NMOSD pathogenesis (32). In the present study, we demonstrated for the first time that the

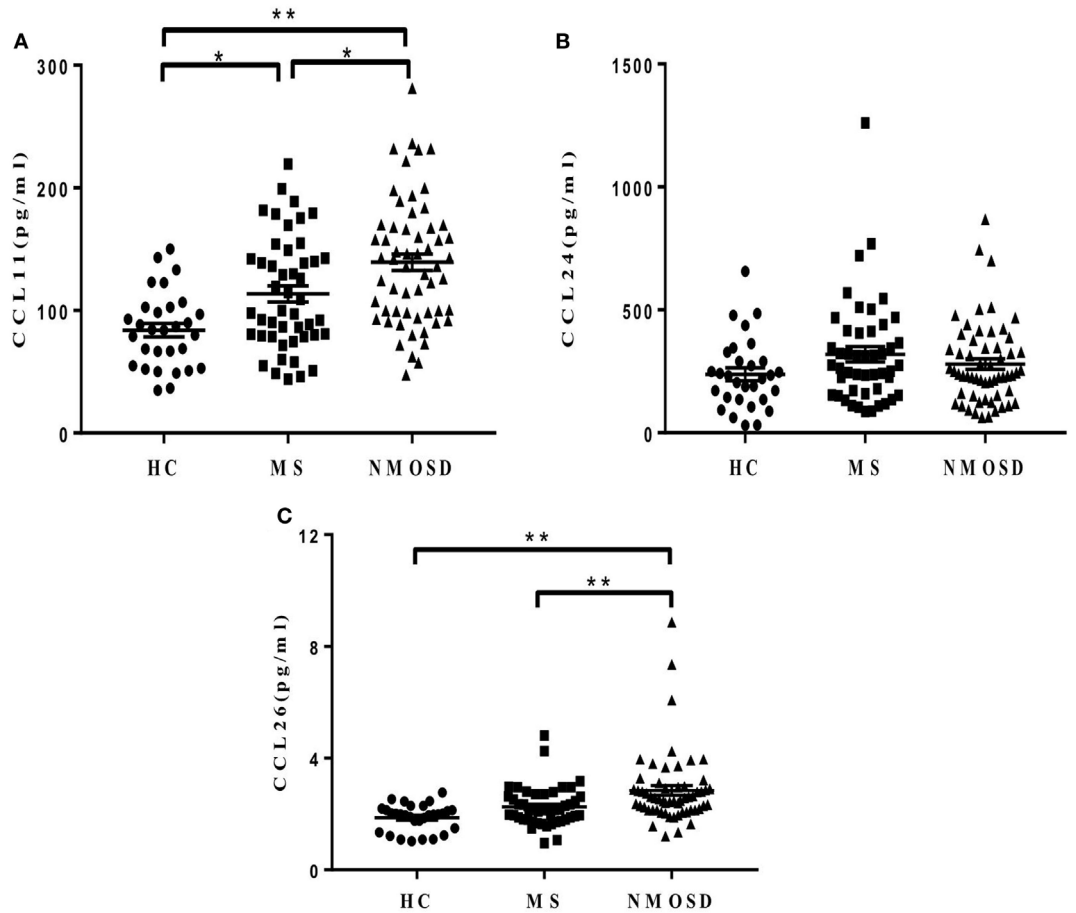

FIGURE 2 | Plasma levels of eotaxins (CCL11, CCL24, and CCL26). **(A,C)** Different plasma levels of CCL11 and CCL26 levels in healthy control (HC), multiple sclerosis (MS) patients, and neuromyelitis optica spectrum disorder (NMOSD) patients (mean ± SE), and **(B)** no significant differences of CCL24 levels were found among the three groups (mean ± SE) ($P = 0.18$). Kruskal–Wallis *H*-test and Dunn's *post hoc* analysis were used. *$P < 0.05$, **$P < 0.01$.

TABLE 2 | Adjusted regression coefficients (β) of CCL13, CCL11, and CCL26 levels with relapse times, ARR, and EDSS scores as outcomes in neuromyelitis optica spectrum disorder patients.

Chemokines	Outcomes	Adjusted β	R^2	*P*-values	Adjusted for
CCL13	Relapse times	0.571	0.553	0.000	Age, disease duration
	ARR	0.002	0.331	0.985	Disease duration
	EDSS	0.007	0.179	0.959	Age
CCL11	Relapse times	0.015	0.274	0.903	Age, disease duration
	ARR	−0.128	0.346	0.281	Disease duration
	EDSS	0.051	0.181	0.700	Age
CCL26	Relapse times	−0.032	0.274	0.803	Age, disease duration
	ARR	0.017	0.331	0.891	Disease duration
	EDSS	−0.031	0.180	0.820	Age

ARR, annual relapse rate; EDSS, expanded disability status scale.

level of the MCP-4 protein is elevated in the sera of patients with NMOSD. This elevation may in turn activate DCs and Th17 cells, especially eosinophils, which could worsen inflammation and lead to recurrence (33). In addition, the association of CCL13 levels with the number of relapses was analyzed, and the results showed a significant positive relationship between them. This finding indicated that CCL13 may play an extensive pathological role during remission in NMOSD. On the basis of these findings, we suggest that blocking the actions of CCL13 might serve as a novel strategy for the generation of agents with anti-inflammatory activity during remission in patients with NMOSD.

Other MCPs that bind with CCR1 and CCR2 to achieve signal cascade were also assayed in the present study. The results showed no significant changes in CCL2 and CCL7 levels during remission

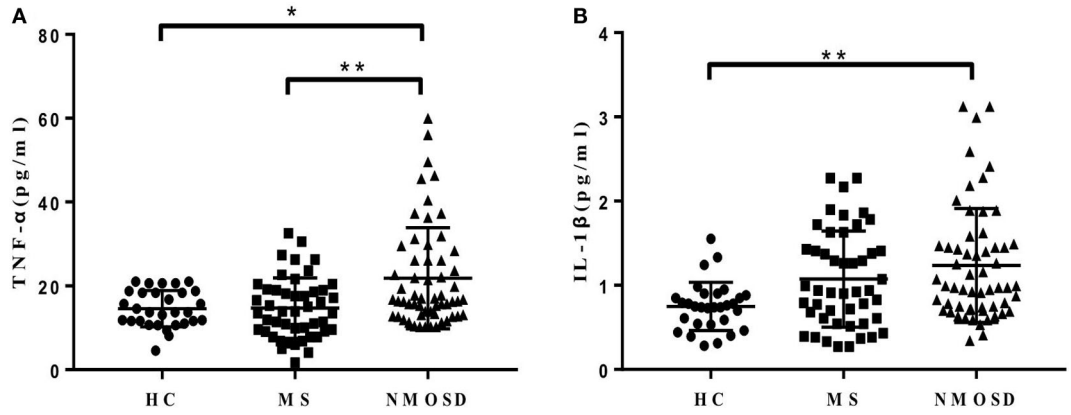

FIGURE 3 | **(A,B)** Plasma levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-lβ) in healthy control (HC), multiple sclerosis (MS) patients, and neuromyelitis optica spectrum disorder (NMOSD) patients. Kruskal–Wallis *H*-test and Dunn's *post hoc* analysis were used. $^{*}P < 0.05$, $^{**}P < 0.01$.

in patients with NMOSD. Further, the CCL7 levels were too low to measure in most patients, which is in line with the findings of most studies (34).

Several studies identified higher levels of eotaxins in the CSF and serum of patients with NMOSD than those in MS patients during acute attacks and concluded that this may be responsible for eosinophil migration across the BBB (10, 35); however, few studies focus on eotaxins levels during remission. The present study showed that plasma CCL11 and CCL26 were upregulated in patients with NMOSD during remission, implying that CCL11 and CCL26 may persistently participate in eosinophil activation, recruitment, and amplification in the pathogenesis of NMOSD after acute attacks. However, CCL24, which is also a strong chemoattractant for eosinophils, was not upregulated during the remission stage. Furthermore, no correlation was found between CCL11 levels, as well as CCL26, and clinical characteristics of patients with NMOSD.

Moreover, TNF-α and IL-1β were examined in this study for their ability to stimulate the release of multiple cytokines including CCL13 and eotaxins through the extracellular signal-regulated kinase cascade (18, 36–40). The results showed that the levels of both these inflammatory markers were elevated in the plasma of patients with NMOSD during remission. This indicated that TNF-α and IL-1β may be involved in the pathogenesis NMOSD mediated by CCL13, CCL11, and CCL26.

Collectively, the findings of the present study indicated that CCL13, CCL11, and CCL26 were upregulated by TNF-α and IL-1β, and this in turn led to the activation of eosinophils. This may have worsened the inflammation, leading to relapses in patients with NMOSD during remission.

ETHICS STATEMENT

Ethics approval for this study was obtained from the ethics committee of the Beijing Tiantan Hospital Affiliated to the Capital Medical University in China (No. KY2015-003-02). Prior to participation, all patients and healthy controls provided written informed consent.

AUTHOR CONTRIBUTIONS

YT, TY, and YF conceived and designed the study. YT, TY, JW, and TZ performed the MILLIPLEX® map human High Sensitivity Cytokine/Chemokine Panels tests, and participated in evaluating the EDSS scores of patients and collecting blood samples. LW, YK, and CC performed the statistical analysis. YF and LW revised the manuscript. All authors reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fneur.2018.00044/full#supplementary-material.

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Imbalance of Circulating T_h17 and Regulatory T Cells in Alzheimer's Disease: A Case Control Study

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The neuropathological hallmarks of Alzheimer's disease (AD), i.e., neuritic plaques and neurofibrillary tangles, consist of beta amyloid peptides (Aβ) and hyperphosphorylated Tau. These are accompanied by reactive microglia and astrocytes in the vicinity of the neuritic plaques and by changes to the peripheral immune system, e.g., an increase of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the peripheral blood. To address a potential involvement of peripheral T helper cell (T_h) subsets in AD, we conducted a case control study with 54 individuals with AD dementia (n = 14), with mild cognitive impairment (MCI) due to AD (MCI_{AD}, n = 14), with MCI unlikely due to AD (MCI_{other}, n = 13), and controls without cognitive impairment (controls, n = 13). The proportions of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells, $CD3^+CD8^-IL\text{-}17A^-IFN\gamma^+$ T_h1 cells, and $CD4^+CD127^{low}CD25^+$ regulatory T cells (T_{regs}) were assessed by flow cytometry. In addition, the correlations of the proportions of T_h subsets to cerebrospinal fluid biomarkers were studied. $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells were significantly increased in subjects with MCI_{AD} compared to age- and sex-matched subjects with MCI_{other} and controls (MCI_{AD} mean = 1.13, SD = 0.77; MCI_{other} mean = 0.58, SD = 0.28; and controls mean = 0.52, SD = 0.22; p = 0.008). The proportion of $CD4^+CD127^{low}CD25^+$ T_{regs} was not altered between the different groups, but it significantly positively related with the levels of total Tau and pTau181 ($r_{Treg|totalTau}$ = 0.43, p = 0.021, n = 28; $r_{Treg|pTau181}$ = 0.46; p = 0.024, n = 28) in subjects with AD but not in nonAD controls ($r_{Treg|totalTau}$ = −0.51, p = 0.007, n = 26). The increase of circulating $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells in the early stages of AD and the association of $CD4^+CD127^{low}CD25^+$ T_{regs} with neurodegeneration marker Tau may indicate that the adaptive immune system relates to neuropathological changes in AD.

Keywords: T_h17, regulatory T cell, T_h1, Alzheimer's disease, mild cognitive impairment, Tau, amyloid beta

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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. The neuritic plaques and neurofibrillary tangles are the neuropathological hallmarks of AD. They are accompanied by activated, cytokine-overexpressing microglia and astrocytes in the vicinity of the diffuse and neuritic plaques at early stages of progression in AD brain and by an increase of pro-inflammatory cytokines in the peripheral blood, such as IL-1β, IL-6, and TNF-α (1–6).

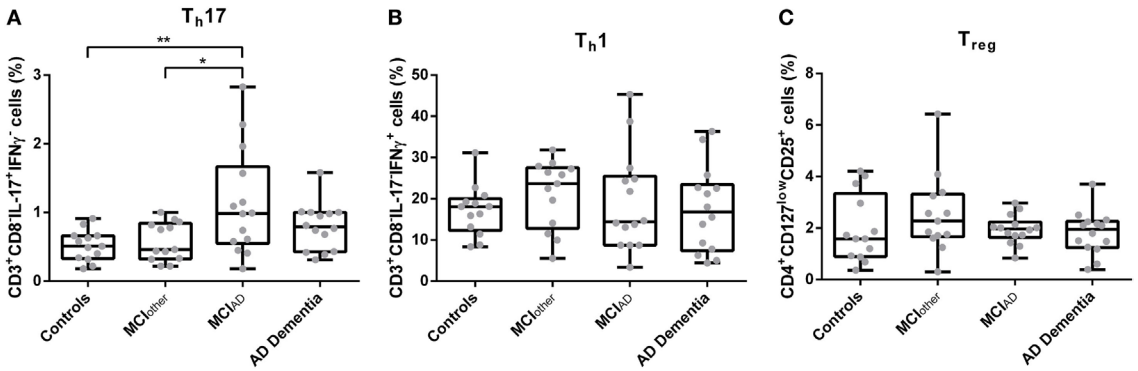

FIGURE 1 | Percentages of $CD3^+CD8^-IL\text{-}17^+IFN\gamma^-$ T_h17 cells **(A)**, $CD3^+CD8^-IL\text{-}17^-IFN\gamma^+$ T_h1 cells **(B)**, and $CD4^+CD127^{low}CD25^+$ T_{regs} **(C)** as assessed with flow cytometry from donor derived cryopreserved peripheral blood mononuclear cells were compared between controls, mild cognitive impairment $(MCI)_{other}$, MCI_{AD}, and Alzheimer's disease (AD) dementia. Data are summarized as combined box- and scatter plots (*p*-values from *post hoc* tests are shown $^*p < 0.05$, $^{**}p < 0.01$).

The blood–brain barrier (BBB) is impaired in patients with AD (7). This may facilitate the cytokine crosstalk between microglia and astrocytes with peripheral blood mononuclear cells (PBMCs) and the migration of PBMCs through the BBB. There is increasing evidence that blood or bone marrow-derived monocytes may either promote or inhibit chronic neuroinflammation in AD after crossing the BBB comparable with resident microglia (8, 9). Similarly, increased percentages of lymphocytes, both $CD8^+$ cytotoxic T-cells and $CD4^+$ T helper (T_{helper}) cells, have been observed in brain parenchyma of patients with AD (10).

Primarily based on murine models, various T_h cell lineages, including T_h1, T_h17, and regulatory T cells (T_{regs}), have been described to be associated with AD (11–20). The T_h1 lineage is promoted by IL-12, is characterized by the transcription factor T-bet and by the secretion of IFNγ (20–22). T_h17 cells are elevated in various autoimmune diseases and diseases associated with chronic inflammation (23, 24). The differentiation of the T_h17 lineage depends on IL-12p40/IL-23p19 and cytokines reportedly increased in AD, i.e., TGF-β, IL-1, and IL-6, and is inhibited among others by IFNγ and IL-4 (25, 26). T_h17 cells are characterized by the secretion of IL-17 and typically express the transcription factor RORγt. IL-17A, which forms homodimers or heterodimers with IL-17F, acts synergistically with other pro-inflammatory cytokines and recruits neutrophils and monocytes to the sites of inflammation (25). High levels of TGF-β and IL-2, however, facilitate the differentiation of T_h cells into regulatory T-cells (T_{regs}), which dampen pro-inflammatory responses. The influence of T_{regs} on neuroinflammation has been extensively studied in mouse models of experimental autoimmune encephalomyelitis (EAE), i.e., a commonly used model for multiple sclerosis (27). In this model, T_{reg} cells are able to dampen deleterious pro-inflammatory effects, e.g., demyelination, and promote myelin regeneration directly *via* increased CCN3 expression and by immunomodulation among others *via* suppressing the Th1- and Th17 cell-mediated immune response (28–30). There is a great variety of $CD4^+$ T_{regs}, usually identified by the expression of $CD25^+$ and the intracellular transcription factor Foxp3 or the expression of CD25 combined with low levels of CD127 (31, 32).

In the revised criteria for AD by the diagnostic guidelines for AD of the National Institute on Aging-Alzheimer's Association (NIA-AA) workgroups, the diagnosis of AD is no longer merely based on clinical examination, neuropsychiatric tests, and exclusion of other causes for dementia. In the revised criteria, the diagnostic procedures include the detection of amyloidopathy in terms of a decrease of amyloid beta (Aβ) 42 in cerebrospinal fluid (CSF) or a positive amyloid-PET and of neurodegeneration reflected by an increase of Tau and of hyperphosphorylated Tau (pTau) or glucose hypometabolism in FDG-PET (33, 34). The detection of the CSF biomarkers Tau and pTau does not only provide a higher sensitivity and specificity for the diagnosis of AD dementia but also indicate a higher rate of progression from mild cognitive impairment (MCI) to AD dementia (35, 36).

Based on the increase of pro-inflammatory cytokines and T_h subsets found in AD brains, this study assesses the proportions of $CD8^+CD3^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells, $CD8^+CD3^-IL\text{-}17A^-IFN\gamma^+$ T_h1 cells, and $CD4^+CD127^{low}CD25^+$ T_{regs} in cryopreserved PBMCs of subjects with Alzheimer's dementia (AD dementia), MCI due to AD (MCI_{AD}), MCI unlikely due to AD (MCI_{other}), and subjects without cognitive impairment (controls). The associations between the percentages of the T_h subsets and CSF biomarkers are investigated.

MATERIALS AND METHODS

Study Population

The study protocol was approved by the clinical ethics committee of the University of Erlangen-Nuremberg. Patients and their authorized legal representatives provided written informed consent after receiving a complete description of the study. Fifty-four individuals with AD dementia ($n = 14$), with MCI_{AD} ($n = 14$), MCI_{other} ($n = 13$), and controls ($n = 13$) frequency matched for sex, age, and years of education were enrolled in this case control study.

Each participant was examined by a psychiatrist with advanced training in neuropsychiatry and dementia research according to the protocol of the German version of Consortium to Establish a

Registry for AD (CERAD) clinical assessment battery. All participants with AD as well as 19 non-demented controls were assessed with the German version of the CERAD neuropsychological battery (CERAD-NB), trail making test part A and B (TMT-A and -B), and single letter Phonemic fluency (CERAD-NB+). Two subjects with AD dementia had severe cognitive deficits, therefore, only the MMSE was performed. TMT-A or TMT-B were dismissed, when the time limit had been exceeded. All subjects were assessed with SPECT and CSF dementia diagnostics [analysis of Aβ 42, Aβ 40, Aβ42/Aβ40 ratio, Tau, and phospho-Tau (pThr181, pTau)]. To exclude other potential causes for dementia, routine laboratory analyses (e.g., blood count, measurement of serum electrolytes, urea, creatinine, TSH, vitamin B12, folate, and CRP), routine CSF analyses, and MRI of the head were performed. Participants with focal brain lesions on MRI were excluded from this study. Classification into possible and probable AD dementia and MCI_{AD} was performed by diagnostic guidelines for AD of the NIA-AA workgroups (33, 34). When the decrease of Aβ 42 was substituted by a decrease of Aβ42/Aβ40 ratio for the discrimination between possible and probable AD, all 13 subjects with AD dementia fulfilled the criteria for probable AD (37). MCI probable due to AD (MCI_{AD}) was similarly defined by the decrease of Aβ42/Aβ40 ratio and increase of Tau/pTau181, while all other subjects with MCI (MCI_{other}) had negative CSF biomarkers.

The severity of concomitant depressive symptoms was monitored by the Beck's depression inventory II or the geriatric depression scale. The donors of the samples were enrolled in the study during the initial diagnosis, which is why none of them received treatment with acetylcholinesterase inhibitors or memantine at the time of sample acquisition.

CSF Biomarker Diagnostics

The concentrations of Aβ 40 and Aβ 42 in CSF were measured with commercially available ELISAs from IBL International (Hamburg, Germany). Total Tau and pTau181 in CSF were assessed with an ELISA from Fujire-bio Europe (Gent, Belgium).

Isolation of PBMCs

Whole blood was collected in EDTA containing S-Monovettes® (Sarstedt, Nümbrecht, Germany). PBMCs were isolated by density gradient centrifugation with Biocoll separatin solution (Merck, Darmstadt, Germany) and cryopreserved with Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, Schwerte, Germany) in RPMI (Gibco, now Thermo Fisher Scientific) with 20% v/v FBS and 10% v/v DMSO (Roth, Karlsruhe, Germany) and stored in liquid nitrogen.

Flow Cytometry

The following fluorochrome-conjugated monoclonal antibodies were purchased from commercial vendors: PE/Dy647 conjugated anti-CD3 (clone: MEM-57, Immunotools, Friesoythe, Germany), APC conjugated anti-CD4 (clone: EDU-2, Immunotools), PE conjugated anti-CD127 APC (clone: eBioRDR5, eBioscience, now Thermo Fisher Scientific, Frankfurt, Germany), IL-17A (clone: eBio64DEC17, eBioscience), FITC conjugated anti-CD25 (clone: HI25a, Immunotools), and Alexa488 conjugated anti-IFNγ (clone: B27, BD Biosciences; Heidelberg, Germany). PE conjugated IgG1κ (eBioscience) and FITC conjugated IgG1 (clone: PPV-06, Immunotools) served as isotype controls.

Cryopreserved PMBCs were thawed, and the number of viable leukocytes was determined by the CASY® TT Cell Counter + Analyzer (OLS Omni Life Science, Bremen, Germany). Cells were resuspended in RPMI1640 with 10% v/v FBS superior (Merck Millipore, Darmstadt, Germany)/1% v/v penicillin (10,000 U/ml)/streptomycin (10 mg/ml) (S/P, Gibco)/20 µg/ml DNAse (Sigma-Aldrich, Taufkirchen, Germany), spun with 500 *g* for 5 min, and cultivated in RPMI1640/10% v/v FBS/1% v/v S/P overnight in an incubator at 37°C and 5% CO_2. For surface staining, 0.2×10^6 cells were washed twice in PBS/0.1% v/v FBS and stained with combinations of APC conjugated anti-CD4, PE conjugated anti-CD127, FITC conjugated anti-CD25, and/or FITC conjugated IgG1 (1:200, each). For the staining of intracellular cytokines, 0.2×10^6 cells were stimulated with RPMI/10% FBS/1% S/P with 20 µg/ml DNAse, phorbol myristate acetate (PMA) 50 ng/ml (Sigma-Aldrich), and ionomycin 500 ng/ml (Sigma-Aldrich). After 1 h, 5 µg/ml Brefeldin (Sigma-Aldrich) and 5 µg/ml Monensin (BD Biosciences) were added, and the cells were incubated for an additional 5 h. Cells were washed twice in PBS/0.1% v/v FBS, stained with PE/Dy647 conjugated anti-CD3 and APC conjugated anti-CD8 for 20 min at room temperature in the dark. Permeabilization, fixation, and staining of intracellular cytokines with Alexa488 conjugated anti-IFNγ and PE conjugated anti-IL-17A or PE conjugated IgG1κ (1:200, each) were performed with Cytofix/Cytoperm® Plus Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions. T_h cells from PMA and ionomycin stimulated cultured PBMCs were defined by a combination of FSC and SSC characteristics and CD3/CD8 stain, as CD4 was extensively downregulated under PMA and ionomycin stimulation as previously reported (38–40).

Analyses were performed using a Partec CyFlow® Space flow cytometer (Sysmex Europe, Norderstedt, Germany) and Flomax® operating sofware v2.9 (Sysmex Europe) and Kaluza Flow Cytometry Analysis Software v1.5.20207.16062 (Beckman Coulter Life Sciences, Krefeld, Germany). Viable lymphocytes were identified by their characteristics in forward scatter (FSC) and side scatter (SSC) and the percentage of viable lymphocytes in each preparation was determined by the CASY cell counting technology. The viable lymphocyte gate was based on Annexin V and propidium iodide stain. Representative dot plots are given in Figure S3 in Supplementary Material. Isotype control antibodies and single-stained samples were used periodically for color compensation and to check the settings.

Statistical Analysis

Scores in CERAD-NB+ were converted to *z*-scores. Shapiro–Wilk's test was used to test for normality. Homogeneity of variance was assessed by Levene's test. Group comparisons were performed using Pearson's χ^2 for categorical variables or the Mann–Whitney *U* test or Kruskal–Wallis test followed by Dunn's multiple comparison test in case a significant effect was observed for ordinal or not normally distributed interval variables. The ANOVA and for groups with inhomogenous variances the Brown–Forsythe test were employed for normally distributed interval variables followed by Tukey B or Dunn–Bonferroni correction in case a

significant effect was observed. Pearson correlation was used to examine the relationships between variables of interest. Data analysis was performed using the SPSS statistical package (version 20.0; SPSS, Chicago, IL, USA). Quartiles are indicated as follows: 1st quartile = Q1; 3rd quartile = Q3; significance levels are indicated as follows: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; and ns, not significant.

RESULTS

Description of the Study Population

The groups of subjects with AD dementia, MCI probably due to AD (MCI_{AD}), MCI with negative CSF biomarkers (MCI_{other}), and without cognitive impairment (controls) did not differ statistically in gender [$\chi^2(3) = 1.261$, $p = 0.738$, $n = 54$], age [$F(3,54) = 2.338$, $p = 0.085$, $n = 54$], or education ($H = 2.718$, d.f. = 3, $p = 0.437$, $n = 47$). A detailed listing of the levels of CSF biomarkers and z-scores of the CERAD-NB$^+$ is given in **Table 1**.

Circulating $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 Cells Were Increased in Participants With MCI_{AD} Compared to Subjects Without Cognitive Impairment and With MCI_{other}

The gating strategy for the analysis of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells, $CD3^+CD8^-IL17A^-IFN\gamma^+$ T_h1, and $CD4^+CD127^{low}CD25^+$ T_{regs} from cultured cryopreserved PBMCs is depicted in Figure S1 in Supplementary Material. Doublet exclusion by FSC-H and FSC-A scatter did not relevantly alter the percentages of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells, $CD3^+CD8^-IL17A^-IFN\gamma^+$ T_h1 and $CD4^+CD127^{low}CD25^+$ T_{regs} (Figure S3 in Supplementary Material). The proportion of peripheral PMA/ionomycin treated $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells was significantly altered between subjects with AD dementia, with MCI_{AD}, with MCI_{other} and in controls [F^*(d.f.1 = 3, d.f.2 = 24.339) = 5.314, $p = 0.008$, $n = 54$] (**Figure 1**). The highest abundance of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells in relation to $CD3^+CD8^-$ cells was detected in the group with MCI_{AD} (mean = 1.13, SD = 0.77), followed by AD dementia (mean = 0.79, SD = 0.33), MCI_{other} (mean = 0.58, SD = 0.28), and controls (mean = 0.52, SD = 0.22). The subsequent Dunn–Bonferroni test showed that the proportion of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells in MCI_{AD} was significantly elevated compared to controls ($z = -0.61231$, $p = 0.007$; $d_{Cohen} = 1.07$) and subjects with MCI_{other} ($z = -0.55007$, $p = 0.019$; $d_{Cohen} = 0.95$), indicating that circulating $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells are elevated especially in early stages of AD. Representative dotplots of $CD3^+CD8^-$ and $CD4^+$ lymphocytes are given in Figure S2 in Supplementary Material.

The abundances of $CD3^+CD8^-IFN\gamma^+IL\text{-}17A^-$ T_h1 cells and $CD4^+CD127^{low}CD25^+$ T_{regs} from peripheral blood did not differ statistically significantly between the groups [$F(3,50) = 0.498$, $p = 0.686$, $n = 54$ and $F^*(3,50) = 1.106$, d.f.1 = 3, d.f.2 = 34.213, $p = 0.360$, $n = 54$, respectively]. Similarly, the mean fluorescence intensities of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells, $CD3^+CD8^-IFN\gamma^+IL\text{-}17A^-$ T_h1 cells, and $CD4^+CD127^{low}CD25^+$ T_{regs} did not significantly vary between AD dementia, MCI_{AD}, MCI_{other}, and controls [$F(3,50) = 0.274$, $p = 0.844$, $n = 54$; $H = 1.290$, d.f. = 3, $p = 0.731$, $n = 54$; and $H = 1.669$, d.f. = 3, $p = 0.644$, $n = 54$, respectively]. Analyses of control parameters showed a significant difference in the serum cholesterol levels between the groups. The other control parameters, i.e., leukocyte count, levels of CRP, of TSH, of folate, of vitamin B12, of urea, of creatinine, and of fastening glucose did not differ statistically significantly in multivariate analyses as given in Table S1 in Supplementary Material.

The Proportion of Circulating $CD4^+$ $CD127^{low}CD25^+$ T_{regs} Cells Was Positively Related to Total Tau, pTau181, and Aβ 40 in the AD but Not in the nonAD Group

Correlations of the proportions of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells, $CD3^+CD8^-IFN\gamma^+IL\text{-}17A^-$ T_h1 cells, and $CD4^+CD127^{low}$ $CD25^+$ T_{regs} cells with total Tau, pTau181, Aβ 42, Aβ 40, and Aβ42/Aβ40 ratio were analyzed in subjects with probable AD and in subjects with negative AD biomarkers (nonAD group).

In the AD group, the proportions of peripheral $CD4^+$ $CD127^{low}CD25^+$ T_{regs} were statistically significantly positively related to the amount of the neuronal injury marker total Tau in CSF ($r = 0.433$, $p = 0.021$, $n = 28$), to pTau ($r = 0.462$, $p = 0.024$, $n = 28$), and to Aβ 40 ($r = 0.484$, $p = 0.009$, $n = 28$) (**Figure 2**). In the nonAD group on the other hand, total Tau in CSF significantly negatively related to the level of $CD4^+CD127^{low}CD25^+$ T_{regs} ($r = -0.513$, $p = 0.007$, $n = 26$). In addition, the percentages of $CD4^+CD127^{low}CD25^+$ T_{regs} were only statistically significantly positively associated with the ratio of Aβ42/Aβ40 in the nonAD group ($r = 0.450$, $p = 0.021$, $n = 26$). Collectively, the percentage of $CD4^+CD127^{low}CD25^+$ T_{regs} in the AD group positively related to the level of neurodegeneration in means of increased levels of Tau/pTau181 and amyloidopathy in means of decrease of Aβ42/Aβ40 ratio, while in the nonAD group an inverse relation was observed.

The percentages of $CD4^+CD127^{low}CD25^+$ T_{regs} did not correlate with age ($r_{AD} = 0.01$, $p_{AD} = 0.98$ and $r_{nonAD} = 0.02$, $p_{nonAD} = 0.91$) or education ($r_{AD} = 0.25$, $p_{AD} = 0.21$ and $r_{nonAD} = 0.19$, $p_{nonAD} = 0.41$). There was no significant difference in the proportion of $CD4^+CD127^{low}CD25^+$ T_{regs} between males and females [$\chi^2(25)_{AD} = 28.000$, $p_{AD} = 0.411$, $n_{AD} = 28$ and $\chi^2(25)_{nonAD} = 27.000$, $p_{nonAD} = 0.356$, $n_{nonAD} = 27$]. The percentage of $CD3^+CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 was statistically significantly positively related to pTau181 in the nonAD group ($r = 0.480$, $p = 0.013$, $n = 26$).

DISCUSSION

In this case control study, the proportion of peripheral $CD3^+$ $CD8^-IL\text{-}17A^+IFN\gamma^-$ T_h17 cells was significantly elevated in subjects with MCI_{AD} compared to controls and subjects with MCI_{other}. Together with the observation, that the proportion of $CD4^+CD127^{low}CD25^+$ T_{regs} was significantly positively related with the level of neurodegeneration markers pTau181 and total Tau in patients with AD but not in controls, the results indicate that these T_h cell lineages might be associated with the neurodegeneration in AD.

TABLE 1 | Clinical data including cerebrospinal fluid biomarkers and *z*-scores of the CERAD-NB+ of the investigated groups.

	Controls				Mild cognitive impairment $(MCI)_{other}$				MCI_{AD}				Alzheimer's disease dementia				*p*-Value
	Mean ± SD			*n*	Mean ± SD			*n*	Mean ± SD			*n*	Mean ± SD			*n*	
Age (years)	61.1 ± 5.9				63.6 ± 7.7				66.7 ± 6.6				67.0±6.5				0.085[b]
Education (years)	15.13 ± 3.8			[d]	14.50 ± 3.8			[d]	12.2 ± 4.1				13.2±4.1				0.437[c]
Sex (♀/♂)				(4/9)				(3/10)				(5/9)				(6/8)	0.738[a]
	Q1	Median	Q3		Q1	Median	Q3		Q1	Median	Q3		Q1	Median	Q3		
Tau (pg/ml)	167.0	247.0	284.5		185.0	226.0	260.0		486.8	597.5	989.5		508.0	660.0	964.3		
pTau181 (pg/ml)	25.5	35.9	45.2		38.9	43.5	51.1		88.3	99.3	117.3		82.5	98.3	126.3		
Beta amyloid 40 (pg/ml)	9,591	12,911	17,678		9,265	13,156	14,334		14,029	15,374	20,052		13,537	14,664	16,846		
Beta amyloid 42 (pg/ml)	704.7	872.4	1,226.3		689.4	900.8	1,085.8		488.6	580.0	677.9		496.1	598.8	645.0		
Ratio Aβ 42/Aβ 40 ratio Aβ 42/Aβ 40	0.069	0.075	0.078		0.069	0.074	0.078		0.030	0.036	0.043		0.034	0.038	0.042		
MMSE	−1.3	−0.3	0.6		−3.1	−1.5	−0.3		−2.8	−2.3	−1.6		−5.3	−4.7	−3.6		
Semantic verbal fluency test	−0.5	0.2	0.8	[d]	−2.2	−1.7	−0.8	[d]	−1.6	−1.2	0.3		−2.4	−2.3	−1.1	[d]	
Boston naming test	0.3	0.5	0.7	[d]	−0.7	0.3	0.4	[d]	−1.8	−0.4	1.2		−2.8	−1.8	−1.2	[d]	
Word list memory	0.0	0.5	1.0	[d]	−2.3	−1.3	−0.2	[d]	−3.7	−2.6	−2.0		−5.1	−3.6	−3.3	[d]	
Word list recall	0.3	0.7	1.4	[d]	−1.7	−1.3	−0.4	[d]	−3.6	−2.2	−1.3		−4.3	−3.6	−2.8	[d]	
Constructional practice	0.4	0.5	0.7	[d]	0.2	0.4	0.7	[d]	−1.7	0.4	0.7		−3.7	−2.9	−1.9	[d]	
Recall of constructional praxis	−1.3	0.7	0.9	[d]	−2.0	−1.3	0.0	[d]	−3.5	−1.6	−1.3		−4.3	−3.4	−2.7	[d]	
Phonetic verbal fluency test	−0.1	0.7	2.0	[d]	−1.6	−1.2	−0.4	[d]	−0.9	−0.2	0.7		−3.1	−1.2	−0.4	[d]	
Trail making test A	−0.3	1.0	1.5	[d]	−1.4	−0.5	−0.1	[d]	−1.2	−0.3	0.6		−2.7	−2.1	−1.1	[d]	
Trail making test B	−0.5	0.5	2.2	[d]	−0.9	−0.7	−0.3	[d]	−2.1	−1.6	0.1	[d]				[d]	

n, number of subjects; Q1, first quartile; Q3, third quartile.

p-Values from [a]Person χ^2, [b]one-way ANOVA, [c]Kruskal–Wallis test; [d]data are missing for one or more subjects.

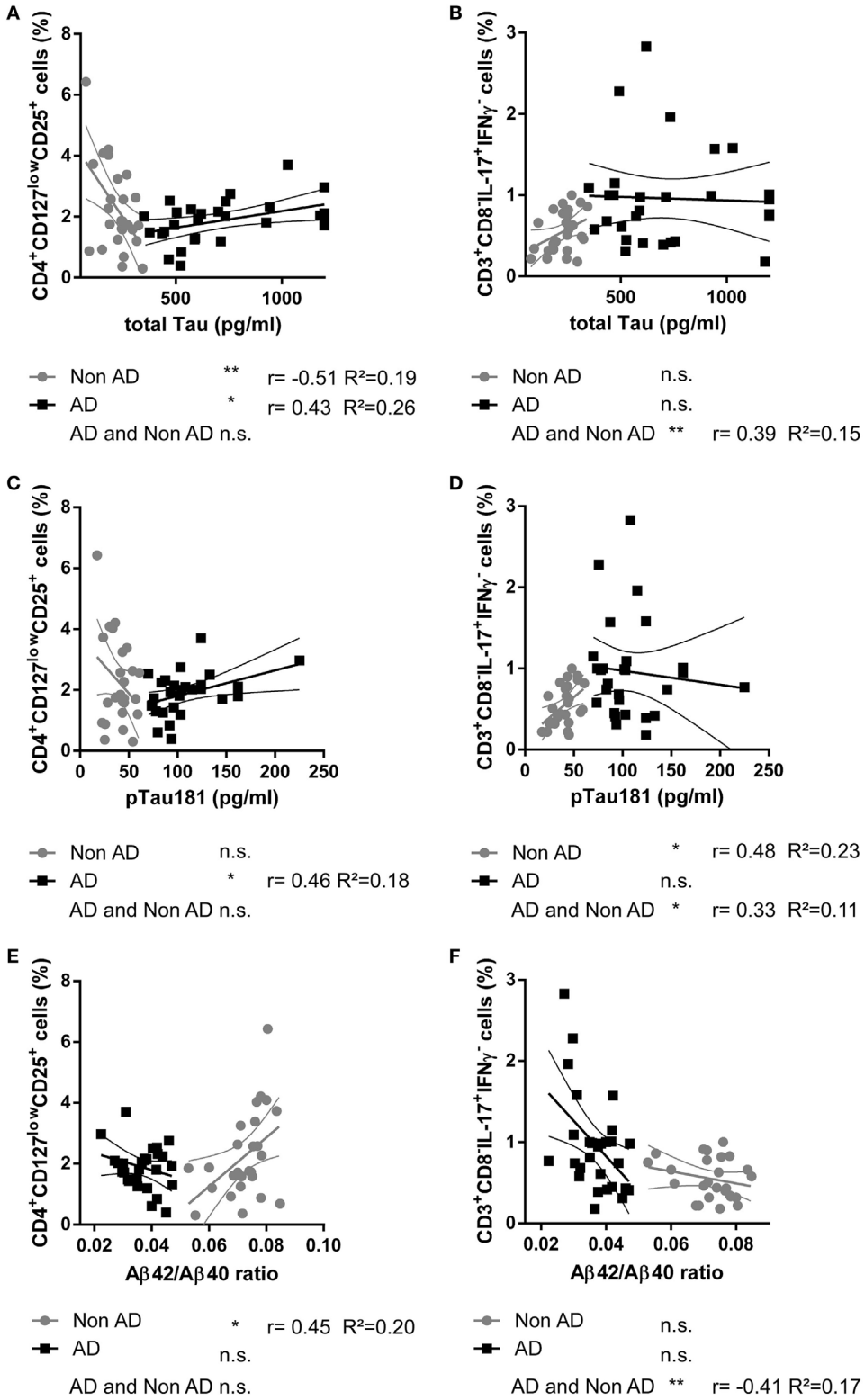

FIGURE 2 | Scatter plots showing the relationship between the cerebrospinal fluid level of total Tau protein [pg/ml; **(A,B)**], pTau181 [pg/ml; **(C,D)**], Aβ42/Aβ40 ratio **(E,F)**, and the percentage of $CD4^+CD127^{low}CD25^+$ T_{regs} **(A,C,E)** and $CD3^+CD8^-IL\text{-}17^+IFN\gamma^-$ T_h17 cells **(B,D,F)** in donors with Alzheimer's disease (AD, black squares) and controls (nonAD, gray dots). The associations in AD group, nonAD group and all subjects combined were investigated with Spearman's correlation. (Scatter plots with the linear regression lines for AD and nonAD groups and their 95% confidence intervals are shown; non-significant = n.s., $^*p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.001$; r: Spearman's correlation coefficient; R^2: coefficient of determination.)

First indications, that the proportion of Th17 cells and RORγt$^+$ cells, respectively, was elevated in Alzheimer's dementia, were reported by Saresella et al. (16) and Agnes et al. (41). Both used the NINCDS ADRA criteria for the diagnosis of Alzheimer's dementia without the detection of the CSF biomarkers. Saresella et al. similarly detected the highest proportions of RORγt$^+$ cell in subjects with MCI, though no differentiation was made between subjects with MCI probably due to AD and MCI unlikely due to AD. An elevation of CCR6$^+$ lymphocytes in 16 patients with AD has been described by Goldeck et al. (42). CCR6$^+$ is a chemokine receptor found on T_h17 cells, subsets of T_{regs}, and dendritic cells and among others is involved in the recruitment of dendritic cells and T cells to the site of inflammation (43). In this study, we were able to differentiate the proportions of Th17 cells between MCI_{AD} and MCI_{other} for the first time. In addition, due to the simultaneous detection of the CSF biomarkers used for the diagnosis of AD dementia and MCI_{AD}, we were able to relate the levels of CSF biomarkers to the percentages of CD3$^+$CD8$^-$IL-17A$^+$IFNγ$^-$ T_h17 and CD4$^+$CD127^{low}CD25$^+$ T_{regs}. In our study, the level of Th17 cells statistically significantly related to amyloidopathy, i.e., the decrease of the ratio of Aβ42/Aβ40. Findings from previous studies indicate that Aβ can directly influence the cytokine expression of Th17 cells, as the disruption of the BBB by hippocampal application of Aβ 42 in rats was followed by an infiltration of T_h17 cells into the brain parenchyma, increased expression of IL-17 and IL-22 in the hippocampus, and elevated concentrations of the two cytokines in both CSF and serum (20). The production of IL-17 from mice splenocytes was elevated after treatment with Aβ 42 and the level of IL-17A and of T_h17 cells in human PBMCs was increased after the stimulation with Aβ 25–35 (13, 19).

The role of T_{regs} in the pathogenesis of AD is a matter of recent debate. In accordance with previous studies, we did not detect a significant difference between CD4$^+$CD127^{low}CD25$^+$ T_{regs} in subjects with AD dementia or MCI_{AD} compared to age-matched controls or MCI_{other} (15, 17). However, Saresella et al. reported an increase of CD4$^+$Foxp3$^+$ T_{regs} in Alzheimer's dementia especially in MCI diagnosed by NINCDS ADRA criteria, Le Page et al. reported an increase of CD4$^+$Foxp3$^+$CD25^{high} cells in amnestic MCI but not in mild Alzheimer's dementia, and Larbi et al. reported an overall decreased frequency of CD4$^+$CD25^{high} T cells in subjects with mild AD dementia when compared with age-matched controls (17, 44, 45).

Our findings suggest that in AD but not in controls the number of CD4$^+$CD127^{low}CD25$^+$ T_{reg} cells was rather related to the level of Tau and pTau181, which predict the rate of cognitive decline in the different stages of AD and correlated with neurofibrillary tangle pathology in the neocortex in AD brains (35, 36, 46, 47). A possible pathomechanism might be that the number of peripheral T_{regs} was elevated in case of increased neurodegeneration in AD to prevent further demyelination and axonal loss similar to the findings in the models of EAE. Observations in a transgenic mouse model of AD (APPPS1 mice) likewise indicated that T_{regs} are able to slow the disease progression and restore cognitive function (48). In addition, an increased suppressive activity of T_{reg} cells in AD and Parkinson's disease has been described (15). However, in 5XFAD mice carrying five mutations associated with early onset familial AD, the depletion of Foxp3$^+$ T_{regs} was accompanied with increased Aβ clearance and reversal of cognitive decline and in a model for neuronal injury in BALB/c/OLA mice, the transfer of CD4$^+$CD25$^+$ T_{regs} worsened the outcome, indicating a deleterious effect of T_{regs} in these models (49, 50). Taken together, the function or loss of function of T_{regs} in AD has still to be determined and further studies are required to identify causal relationship and to provide evidence that the relations to the CSF biomarkers in this study are not due to a common response variable.

Complementing but further complicating the immunological changes in AD, the profile of interleukins supports our study's findings, such as IL-6 and TNF-α, which are commonly elevated in serum in AD, are induced by IL-17 from the T_h17 cells (5, 25). Furthermore, the inhibition of the IL-12/IL-23 pathway, which supports the differentiation of T_h17 cells, attenuated AD pathology and cognitive deficits in an AD mouse model (51). IL-17 has been reported to be increased in AD by several reports; however, Doecke et al. detected on the contrary a significant decrease of IL-17 in plasma from AD patients in a larger study with 151 subjects (52–55). For the level of TGF-β, studies regularly reported increased as well decreased serum levels in AD (56–58). In low doses, TGF-β promotes the differentiation of T_h17 cells, whereas high levels promote T_{regs}, which dampen the pro-inflammatory response of T_h17 cells. Interestingly, low levels of TGF-β were associated with a faster progression from MCI to AD dementia, as it was observed for high levels of total Tau and pTau181 (35, 36, 59).

Nevertheless, this study has limitations, which include the lack of a healthy control sample because of the difficulties associated with collecting CSF from healthy subjects. Instead, patients without cognitive impairment were enrolled as control. Furthermore, the small sample size and a potentially skewed population due to the fact that subjects were enrolled from our memory unit or patients hospitalized in our psychiatric ward may have some bearing on our results. A larger population study will be informative to further elaborate our initial findings.

CONCLUSION

Our clinical data showed an increase of circulating CD3$^+$CD8$^-$ IL-17A$^+$IFNγ$^-$ T_h17 cells in subjects with MCI_{AD} and an association of CD4$^+$CD25$^+$CD127^{low} T_{regs} with the neurodegeneration markers phospho and total Tau, which complements the observations from fundamental research, that the adaptive immune system seems to be involved in the pathogenesis of AD and its neuropathological changes especially in the early stages of the disease. Further studies might provide more insight into disease progression and the interplay between the neuropathological hallmarks of AD and the peripheral immune system.

DATASETS ARE AVAILABLE ON REQUEST

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study protocol was approved by the clinical ethics committee of the University of Erlangen-Nuremberg (project no. 3987). Patients and their authorized legal representatives provided written informed consent after receiving a complete description of the study.

AUTHOR CONTRIBUTIONS

TO designed the study, performed experiments, analyzed the data, and together with JM drafted the manuscript. LT and JH performed experiments and contributed to revision of the manuscript. JK provided reagents and contributed to the interpretation of findings and revision of the manuscript. PS and MH contributed to the interpretation of findings and revision of the manuscript. 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 https://www.frontiersin.org/articles/10.3389/fimmu.2018.01213/full#supplementary-material.

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Peripheral T Cell Depletion by FTY720 Exacerbates Hypoxic-Ischemic Brain Injury in Neonatal Mice

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Hypoxic-ischemic injury to the developing brain remains a major cause of significant long-term morbidity and mortality. Emerging evidence from neonatal brain injury models suggests a detrimental role for peripheral lymphocytes. The immunomodulatory substance FTY720, a sphingosine-1-phosphate receptor agonist, was shown to reduce adult ischemia-induced neurodegeneration through its lymphopenic mode of action. In the present study, we hypothesized that FTY720 promotes neuroprotection by reducing peripheral lymphocytes and their infiltration into the injured neonatal brain. Term-born equivalent postnatal day 9 C57BL/6 mice were exposed to hypoxia ischemia (HI) followed by a single injection of 1 mg/kg FTY720 or vehicle (0.9% sodium chloride). Brain injury, microglia, and endothelial activation were assessed 7 days post HI using histology and western blot. Peripheral and cerebral leukocyte subsets were analyzed by multichannel flow cytometry. Whether FTY720s' effects could be attributed to its lymphopenic mode of action was determined in T cell-depleted mice. In contrast to our hypothesis, FTY720 exacerbated HI-induced neuropathology including loss of gray and white matter structures. While microglia and endothelial activation remained unchanged, FTY720 induced a strong and sustained depletion of peripheral T cells resulting in significantly reduced cerebral infiltration of CD4 T cells. CD4 T cell subset analysis revealed that circulating regulatory and effector T cells counts were similarly decreased after FTY720 treatment. However, since neonatal HI *per se* induces a selective infiltration of Foxp3 positive regulatory T cells compared to Foxp3 negative effector T cells effects of FTY720 on cerebral regulatory T cell infiltration were more pronounced than on effector T cells. Reductions in T lymphocytes, and particularly regulatory T cells coincided with an increased infiltration of innate immune cells, mainly neutrophils and inflammatory macrophages. Importantly anti-CD3-mediated T cell depletion resulted in a similar exacerbation of brain injury, which was not further enhanced by an additional FTY720 treatment. In summary, peripheral T cell depletion by FTY720 resulted in increased infiltration of innate immune cells concomitant to reduced T cell infiltration and exacerbation HI-induced brain injury. This study indicates that neonatal T cells may promote endogenous neuroprotection in the term-born equivalent hypoxic-ischemic brain potentially providing new opportunities for therapeutic intervention.

Keywords: neonatal hypoxia ischemia, birth asphyxia, brain injury, T cells, FTY720, immune cell infiltration, neuroinflammation

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INTRODUCTION

Perinatal asphyxia, resulting in hypoxic-ischemic encephalopathy (HIE), is one of the worldwide leading causes of death and disability in term-born children. In high-income countries, 1–6 per 1,000 newborns suffer from HIE during the perinatal period often resulting in cerebral palsy, epilepsy, visual impairment, and motor-cognitive deficits in later life (1). The only clinically approved therapy is hypothermia, which is, however, only effective in 40–50% of patients and has to be initiated in a very short time window (2).

The development of additional or alternative causative therapies that prevent neuronal damage and promote neurological recovery is highly warranted (3). A particularly promising therapeutic target in neonatal hypoxia ischemia (HI) is the post-hypoxic inflammatory response, which involves a variety of innate and adaptive immune cells migrating across the activated blood–brain barrier to invade the brain parenchyma (4–6). The suggested dynamics of leukocyte infiltration into the injured neonatal brain with a peak of T cell infiltration observed at 1 and 7 days and persistence up to 3 months after injury (4, 6, 7) make them amenable to therapeutic intervention.

While the significance of peripheral immune cells for the development of secondary lesion growth in adults has been well established (8, 9), little is known about the functional relevance of different immune cell subtypes in neonatal brain injury. Splenectomy prior to HI in neonatal rats resulted in significant neuroprotection (10). However, the role of distinct immune cell subsets was not addressed. A more specific approach was performed in a very recent work by the use of lymphocyte deficient $Rag1^{-/-}$ mice that revealed significantly reduced HI brain injury compared to wild-type control mice (11). While the selective contribution of T or B cells to the development of brain injury remained uncertain in the latter study, Albertsson et al. specifically focused on gamma delta T cells demonstrating that depletion of this T cell subset provides neuroprotection in postnatal day 4 mice (12). Taken together, currently available data suggest that interfering with cerebral infiltration of peripheral lymphocytes after neonatal HI is beneficial, a hypothesis until now only tested in pre-term and/or inflammation-induced brain injury models (11–13). Studies in term-born equivalent models focusing on hypoxic-ischemic injury are lacking.

FTY720 is an immunomodulatory sphingosin-1-phosphate analog, approved for the treatment of relapsing–remitting multiple sclerosis (14). A major effector mechanism of FTY720 is reduction of peripheral lymphocytes by blocking egress of lymphocytes from lymphoid organs through agonist-induced receptor internalization leading to reduced lymphocyte counts in the injured brain (15). Despite the huge amount of studies reporting neuroprotection in adult brain ischemia (16–20), only two studies focused on potential protection by FTY720 in perinatal brain injury. Previous own work in a pre-term model of oxygen-induced toxicity revealed protective effects that were directly attributed to protection of oligodendrocyte precursor cells and rather independent of FTY720s' lymphopenic mode of action (21), Yang et al. showed that FTY720 reduces the amount IL-17 producing CD4 T cells in a pre-term model of inflammation-sensitized hypoxic-ischemic brain injury (22). However, the exact mechanisms underlying the protective effects of FTY720 were only partially characterized, e.g., the detailed composition of the CNS immune cell infiltrate and of circulating leukocytes following FTY720 treatment remain unclear.

In the present study, we hypothesized that FTY720 promotes neuroprotection by reducing peripheral T cells and thus infiltration into the injured brain thereby reducing secondary HI-induced brain injury in term-born equivalent mice.

MATERIALS AND METHODS

Animal Care and Group Allocation

Experiments were performed in accordance to the Animal Research: Reporting of *In Vivo* Experiments guidelines with government approval by the State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia. C57BL/6J mice were bred in house and kept under a 12-h light/dark cycle with food and water *ad libitum*. Bodyweight of pups was recorded at postnatal day 9 (P9), P10, P11, P12, and P16. A total of 275 P9 pups ($n = 140$ female and $n = 135$ male) derived from 40 litters were enrolled. Fifty-seven naïve mice (29 female, 28 male) were used to determine lymphocyte populations in the peripheral blood 1, 3, and 7 days after a single FTY720 injection. Fourteen mice (6 female, 8 male) underwent sham surgery. 12 out of 204 animals (6%) exposed to hypoxia-ischemia died during hypoxia. Remaining animals were randomly assigned to two (saline and FTY720) or four (isotype/saline, isotype/FTY720, anti-CD3/saline, and anti-CD3/FTY720) treatment groups by an independent scientist not involved in data acquisition: the first set of HI mice ($n = 32$; 16 female, 16 male) was used to evaluate brain injury and inflammatory responses *via* histology and western blot 1 week after HI. The second cohort of mice ($n = 110$; 57 female, 53 male) was used for quantification of immune cell subtypes *via* flow cytometry. A third set of mice ($n = 50$, 26 female, 24 male) was used to assess the impact of antibody- and FTY720-mediated T cell depletion and of the combined treatment on HI-induced brain injury *via* histology. In total, two saline and four FTY720-treated mice died between 24 h and 7 days after HI.

Neonatal Hypoxia-Ischemia

Hypoxic-ischemic (HI) brain injury was induced as previously described (23, 24). Briefly, the right common carotid artery was occluded through cauterization (high temperature cauter, 1,200°C, Bovie, USA) under isoflurane anesthesia (1.5–4 Vol%, total duration of surgery: 5–7 min) followed by 1 h hypoxia (10% O_2) in an air-tight oxygen chamber (OxyCycler, Biospherix, USA) after 1 h recovery with their dams. Animals were placed on a warming mat (Harvard Apparatus, USA) to maintain nesting temperature during hypoxia (23). Sham-operated were subjected to anesthesia and neck incision only.

FTY720 Treatment and Antibody-Mediated T Cell Depletion

FTY720 (1 mg/kg body weight, Sigma, #SML 0700 dissolved in 0.9% NaCl) was administered intraperitoneally (i.p.) within

20 min after hypoxia. Dose and administration time point was chosen based on previous studies and experimental reports in adult and neonatal brain injury (19–22, 25). An equal volume of 0.9% NaCl (later referred to "saline") served as control. Antibody-mediated T cell depletion was performed according to our previous protocol by i.p. injection of 16 µg/g body weight anti-mouse CD3 (Clone 17 A2, BioXcell, USA) every 48 h (26). To determine whether effects of FTY720 were specifically dependent on T cells, antibody depletion was started 24 h prior to HI and prolonged to the end of the experiment. Control mice received 16 µg/g body weight isotype control antibody (Clone LTF-2, BioXcell) at the same time points.

Tissue Preparation, Histology, and Immunohistochemistry

One week after HI, mice were deeply anesthetized with chloralhydrate (200 mg/kg body weight) and transcardially perfused with ice-cold phosphate buffered saline (PBS). Brains were removed and snap frozen on dry ice. Tissue injury was assessed and scored on cresyl violet stained 20 µm cryostat sections as previously described (23, 27). Briefly, eight regions were scored: the anterior, middle, and posterior cortex, CA1, CA2, CA3, and dentate gyrus of the hippocampus and the striatum. Each region was given a rating from 0 to 3 (0—no detectable cell loss, 1—small focal areas of neuronal cell loss, 2—columnar damage in the cortex or moderate to severe cell loss in the other regions, 3—cystic infarction and gliosis). The sum score from different regions was calculated for each animal resulting in a total maximum score of 24.

Brain tissue loss was determined by measurement of intact areas in ipsilateral and contralateral hemispheres in two sections from the striatal (+0.2 to +0.3 mm from bregma) and two sections from the hippocampal (−1.9 to −2.0 mm from bregma) level using Image J software (NIH, USA). Tissue loss was determined by comparison with contralateral values according to the following equation: [100 − ratio (ipsilateral/contralateral) × 100].

For qualitative assessment of leukocyte infiltration, cryostat sections were stained for the pan-leukocyte marker CD45 as previously described (24). Briefly, tissue sections were thawed and dried at 37°C followed by fixation in ice-cold aceton/methanol (1:1 v/v%) for 10 min at 4°C. Unspecific antibody binding was blocked by incubation with 5% normal goat serum (NGS), 2% BSA, 0.2% Tween 20 in PBS for 1 h at room temperature. Sections were incubated with rat anti-mouse CD45 (1:20, BD Pharmingen, Germany) in 2% NGS, 1% BSA, 0.2% Tween 20 in PBS at 4°C overnight. Antibody binding was visualized by incubation with anti-rabbit Alexa Fluor 488 (1:500, Invitrogen, Germany) for 2 h at room temperature. Nuclei were counterstained with 4′,6-Diamidin-2-phenylindol (Dapi, 100 ng/ml; Molecular Probes, USA). Images were acquired with confocal microscopy (A1plus, Eclipse Ti, with NIS Elements AR software, Nikon, Germany). Confocal z-stacks of 18 µm thickness (z-plane distance 2 µm) large-scale images (stitching) of complete hemispheres were acquired with a 10× objective.

Western Blot

For western blot analysis 200 µm sections of the ipsilateral hemisphere within the range of −2.0 to −2.3 mm from bregma were dissected and homogenized in ice-cold lysis buffer (RIPA, Sigma-Aldrich) containing protease and phosphatase inhibitors (cOmplete, Roche) and 100 mM PMSF (Sigma-Aldrich). The supernatant was collected and processed as previously described (23, 24). Briefly, after determination of protein concentration using the Pierce bicinchoninic acid assay protein assay kit (Thermo Scientific, USA), protein lysates were separated on gradient sodiumdodecylsulphate (SDS) polyacrylamide gels (Mini-PROTEAN TGX Precast Gels, Any kDa, Bio-Rad, Germany) or 12.5% SDS gels and transferred to nitrocellulose membranes (0.2 µm, Amersham, USA) at 4°C overnight. Equal loading of 20 µg/lane and transfer of proteins was confirmed by staining of membranes with Ponceau S solution (Sigma-Aldrich). Nonspecific binding was blocked by incubation in 5% non-fat milk powder, 0.1% Tween in TBS (TBST). Membranes from gradient SDS gels were incubated with the following primary antibodies: rabbit anti-myelin basic protein (MBP, 1:10.000, Covance, USA), mouse anti-microtubuli associated protein-2 (MAP-2, 1:1,000, Sigma-Aldrich), and goat anti-vascular cell adhesion molecule-1 (VCAM-1, 1:10,000, R&D Systems, USA). Membranes of 12.5% SDS gels were incubated with rabbit anti-ionized calcium binding adaptor molecule-1 (Iba-1, 1:1,000, Wako, Japan) and biotinylated goat anti-mouse ICAM-1 (1:10,000, R&D Systems). Both membranes were incubated with rabbit anti-glutaraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1,000, Santa Cruz, CA, USA) as reference protein. Antibody binding was detected by incubation with appropriate peroxidase-conjugated secondary antibodies [all 1:2,000 except of anti-goat horseradish peroxidase (1:10,000), all Dako, Denmark] in blocking solution at room temperature for 1 h; for detection of ICAM-1 the Vectastain ABC-HRP Kit (Vector Laboratories, USA) was used according to the manufacturers' instructions. Antibody binding was revealed by chemiluminescence using the enhanced chemiluminescence prime western blotting detection reagent (Amersham, GE Healthcare Life Science, USA). For visualization and densitometric analysis, the ChemiDocXRS+ imaging system and ImageLab software (Bio-Rad, Germany) were used.

Processing of Peripheral Blood and Brain Tissues for Flow Cytometry

Isolation of single cell suspension for flow cytometry analysis was performed as previously described (28, 29). Briefly, animals were euthanized by i.p. injections of chloralhydrate (200 mg/kg body weight) followed by transcardial perfusion with ice-cold PBS and removal of brains. Brains were dissected and hemispheres divided into ipsilesional and contralesional parts. Two hemispheres were pooled per sample and homogenized through a 70-µm cell strainer (BD Biosciences) by continuous rinsing with 25 ml of cold HEPES-buffered RPMI1640. Samples were centrifuged at 400 × *g* for 10 min at 18°C. The supernatants were discarded and the pellets were resuspended in 15 ml of 37% Percoll in 0.01 N HCl/PBS and centrifuged at 2,800 × *g* for 20 min. Myelin was removed and the remaining cell pellet was washed twice in PBS.

Blood specimens were collected with ethylenediaminetetraacetate (EDTA) coated capillaries (CLINITUBES, Radiometer, Denmark) by snipping the right atrium of the heart immediately prior to intracardiac perfusion *via* the left ventricle. Blood samples were transferred into (EDTA) coated collection tubes (Minicollect, Greiner Bio One, Germany) and stored until further processing for a maximum of 30 min. Erythrocytes were lysed by incubation with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 3 mM EDTA) for 5 min followed by two washing steps with PBS.

Flow Cytometry

Isolated cells were incubated with antibody cocktails for surface staining provided in Table S1 in Supplementary Material for 30 min at 4°C. For analysis of regulatory T cells, surface staining was followed by fixation and permeabilization with Fix/Perm buffer provided with the regulatory T cell staining kit (eBioscience, Germany) according to the manufactures' instructions followed by intracellular staining of Foxp3 for 30 min at 4°C. Leukocyte subsets were identified and differentiated by their antigen expression using multichannel flow cytometry according to our previously established antibody panels (28). Due to inevitable cell loss by tissue processing (e.g., cytotoxic Percoll density gradient centrifugation) and low blood volumes accessible from neonatal mice, separate experiments for analysis of lymphoid cells (panel 1), regulatory T cells (panel 2), and myeloid (panel 3) cells were performed. Viable leukocytes were identified by gating for CD45 positive cells and FVD (fixable viability dye, eBioscience) negative cells. Microglia expressing low to intermediate levels of CD45 were excluded by gating on $CD45^{high}$ cells (30, 31). Identified leukocytes were further divided into lymphoid subsets (panel 1), i.e., B cells (CD19), natural killer cells (NK1.1), CD4 and CD8 T cells ($CD19^-$, $NK1.1^-$ $CD3^+$, $CD4/8^+$). Regulatory T cells (panel 2) were identified as $CD45^{+/high}$, $CD3^+$, $CD4^+$, $Foxp3^+$ cells (Figures S1A,C in Supplementary Material). For analysis of depletion efficiency in anti-CD3-treated animals, CD3 was excluded from gating. With panel 3 (Table S1 in Supplementary Material), we distinguished neutrophils ($lymphocyte^-$, $Ly6G^+$), monocytes ($lymphocyte^-$, $CD115^+$), macrophages ($lymphocyte^-$, $Ly6G^-$, $CD115^-$, $CD11b^+$), and dendritic cells ($lymphocyte^-$, $Ly6G^-$, $CD115^-$, $CD11c^{high}$) (Figure S1B in Supplementary Material). Resident and inflammatory monocyte/macrophage subsets were distinguished according to their Ly6C expression. Data acquisition and analysis were performed on a BD FACS LSRII equipped with FACS Diva software (BD Biosciences). Total cell counts were determined using BD TrueCount beads (BD) as previously described (29, 32). Inter-experimental variations due to isolation procedures were corrected by relating values of ipsilateral hemispheres to values of contralateral hemispheres of the same animals.

Statistical Analysis

All results were expressed as box and whisker plots with median values. Whiskers display the highest and the lowest value of the total data set. For statistical analysis, the GraphPad Prism 6.0 software package (GraphPad Software) was used. Data were tested for Gaussian distribution with the D'Agostino and Pearson omnibus normality test and then analyzed either by ordinal one-way ANOVA or by Kruskal–Wallis (non-parametric) with *post hoc* Bonferroni correction for multiple comparisons or with Dunn multiple comparison tests, respectively. When two groups were compared, unpaired, two-tailed Student's *t*-test or Mann–Whitney test (non-parametric) were applied. In all analyses, $p < 0.05$ was considered statistically significant.

RESULTS

FTY720 Induces Sustained T Cell Depletion in Neonatal Mice

From adult brain injury models it is suggested that FTY720 mainly acts by sequestering circulating lymphocytes in lymphoid organs through internalization of S1P receptors resulting in reduced amounts of circulating and thus of CNS-infiltrated cells (14, 15, 20). Therefore, we first assessed the number of different lymphocyte subsets *via* flow cytometry 1, 3, and 7 days after a single injection of vehicle (saline) or FTY720 (1 mg/kg body weight i.p.) to 9-day-old naïve C57BL/6 mice. FTY720 induced a strong and long-lasting reduction in the amount of peripheral CD4 and CD8 T cells by 80–99% compared to saline-treated mice (**Figure 1**). The amount of B and natural killer cells was not significantly modulated by FTY720 treatment (Figure S2 in Supplementary Material).

Peripheral T Cell Depletion by FTY720 Exacerbates Neonatal Hypoxic-Ischemic Brain Injury

Because of previous reports on the detrimental role of peripheral T cells in neonatal and adult ischemic brain injury (12, 26, 33, 34) we hypothesized that FTY720-induced depletion of circulating T cells ameliorates HI-induced brain injury. Therefore, we analyzed histopathological changes and brain atrophy in cresyl violet stained brain sections 1 week post HI (**Figure 2A**). Neuropathological assessment revealed that overall and local cortical and hippocampal tissue injury was significantly increased by FTY720 treatment (**Figures 2B–D**). In addition, total and cortical HI-induced brain tissue loss was significantly enhanced in FTY720-treated animals compared to saline-treated mice (**Figures 2E,F**). No significant differences were determined for hippocampal atrophy (**Figure 2G**). In the striatum no differences were observed for neuropathology and tissue atrophy (Figure S3 in Supplementary Material), confirming regional vulnerability to HI injury (35, 36) and regional variability to exogenous interventions (23, 37).

For further insight into target structures with respect to sub-acute gray and white matter injury, western blot analyses for MAP-2 and MBP expression at the level of the hippocampus were performed (**Figure 3A**). HI-induced loss of MAP-2 and MBP was significantly aggravated by FTY720 treatment (**Figure 3B**).

FTY720 Does Not Alter Local Inflammatory Responses and Overall Leukocyte Infiltration

To dissect the underlying mechanisms, we evaluated sub-acute neuroinflammatory responses including microglia and endothelial activation and peripheral leukocyte infiltration 1 week after HI. This time point was chosen to directly compare neuropathological

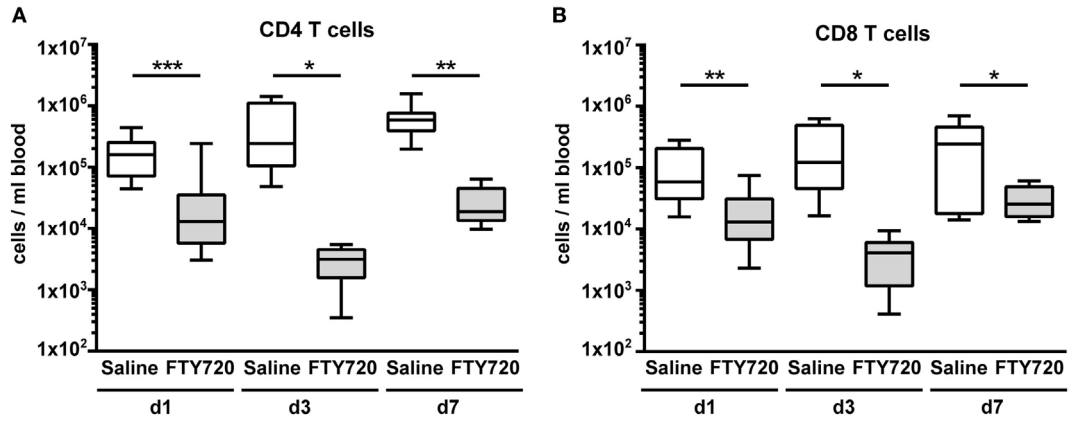

FIGURE 1 | A single injection of FTY720 in postnatal day 9 mice results in a strong and sustained depletion of circulating T cells. Naïve 9-day-old C57BL/6 mice received a single intraperitoneal injection of 1 mg/kg FTY720 (in 0.9% NaCl). Saline-treated animals served as control. One, 3, and 7 days after injection, CD4 T cells **(A)**, CD8 T cells **(B)** were quantified in the blood *via* flow cytometry. Blood samples were collected from the right atrium of the heart and transferred into ethylenediaminetetraacetate coated collection tubes followed by erythrocyte lysis. Cell-specific antigens were stained with appropriate antibodies and viable leukocytes were identified by gating for $CD45^+$ cells and FVD^- and further divided into CD4 and CD8 T cells ($CD19^-$, $NK1.1^-$ $CD3^+$, $CD4/8^+$). FTY720 induced a strong and long-lasting reduction in the amount of peripheral CD4 and CD8 T cells by 80–99% compared to saline-treated mice. $n = 11$–13 for day 1, $n = 8$–10/group for day 3, $n = 7$–8/group for day 7, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Mann–Whitney test for day 1, unpaired *t*-test for day 3 and day 7.

findings with inflammatory responses since previous reports suggested a delayed infiltration of peripheral T cells into the neonatal hypoxic-ischemic brain (4–6, 11). FTY720 treatment induced a slight but not significant increase in microglia activation, assessed by Iba-1 protein expression (**Figures 4A,B**). Protein levels of the adhesion molecules VCAM-1 and ICAM-1, facilitating peripheral immune cell infiltration, were not significantly modulated by FTY720 (**Figures 4A,C,D**). In spite of profound and sustained lymphopenia in the peripheral blood (**Figure 1**), overall HI-induced peripheral leukocyte infiltration into HI-injured brains, qualitatively assessed by immunohistochemistry (**Figure 4E**) and quantified by flow cytometry (**Figure 4F**), was not significantly changed by FTY720 (**Figures 4E,F**). Interestingly, leukocyte infiltration was most prominent in regions mainly affected by the detrimental effect of FTY720, e.g., the hippocampus and cortex (**Figures 4E** and **2A,C,D**), whereas less infiltration was observed in regions not affected by FTY720-induced exacerbation of brain injury, i.e., the striatum (**Figure 4E**; Figure S3 in Supplementary Material).

Systemic T Cell Depletion by FTY720 Leads to Increased Infiltration of Innate Immune Cell Subsets Into the Neonatal Hypoxic-Ischemic Brain

Due to the surprising finding that peripheral T cell depletion by FTY720 results in similar infiltration of peripheral immune cells in general, we performed detailed analyses on the composition of the CNS infiltrate. We detected a significantly reduced amount of infiltrated CD4 T cells after HI in FTY720-treated animals compared to saline-treated mice (**Figure 5A**). CD4 T cell subset analysis revealed a significant reduction of Foxp3 positive regulatory T cells while infiltration of Foxp3 negative effector T cells was not changed in injured hemispheres of FTY720-treated animals (**Figure 5A**). These differences are caused by a selective infiltration of regulatory T cells after neonatal HI which is demonstrated by a 9.1-fold increase in regulatory T cells but only a 1.8-fold increase of effector T cells in ipsilateral hemispheres compared to contralateral parts in saline-treated control mice (**Figure 5A**).

Considering that the overall amount of infiltrated leukocytes was similar between both groups (**Figures 4E,F**), these results suggested an increased abundance of other immune cell subsets in the brain of FTY720-treated animals. Indeed, infiltrated cell numbers of different innate immune cell subsets, such as natural killer cells, neutrophils, dendritic cells, and macrophages (**Figures 5A,B**) were significantly increased in animals with FTY720-induced systemic T cell depletion compared to saline-treated animals. Considering the heterogeneity of monocyte/macrophage phenotypes, we further distinguished between resident and inflammatory cells based on their Ly6C expression (**Figure 5C**). The percentage of $Ly6C^+$ inflammatory cells was significantly increased in the macrophage and monocyte population of T cell-depleted mice (**Figure 5C**). These results suggest that FTY720-induced depletion of T cells in the periphery and the hypoxic-ischemic brain is counteracted by an increased infiltration of innate and particularly inflammatory immune cell subtypes resulting in a similar amount of total leukocytes in the ipsilateral injured hemisphere in both experimental groups (**Figures 4E,F**). This was confirmed by analyzing the percentage of different immune cell subsets in the total leukocyte population in the brain. While FTY720 treatment resulted in a reduction of T cell frequency from 1.6 to 0.5% of total viable leukocytes, a significant increase in neutrophil and dendritic cell percentages from 11.1 to 29.6% ($p < 0.05$) and from 1.9 to 3.5% ($p < 0.05$), respectively, was detected in ipsilateral hemispheres.

To clarify whether the increased amount of innate immune cells in the hypoxic-ischemic brain was caused by in increased transmigration or rather reflected changes in the periphery, we quantified immune cell subtypes in the blood 1 week after HI.

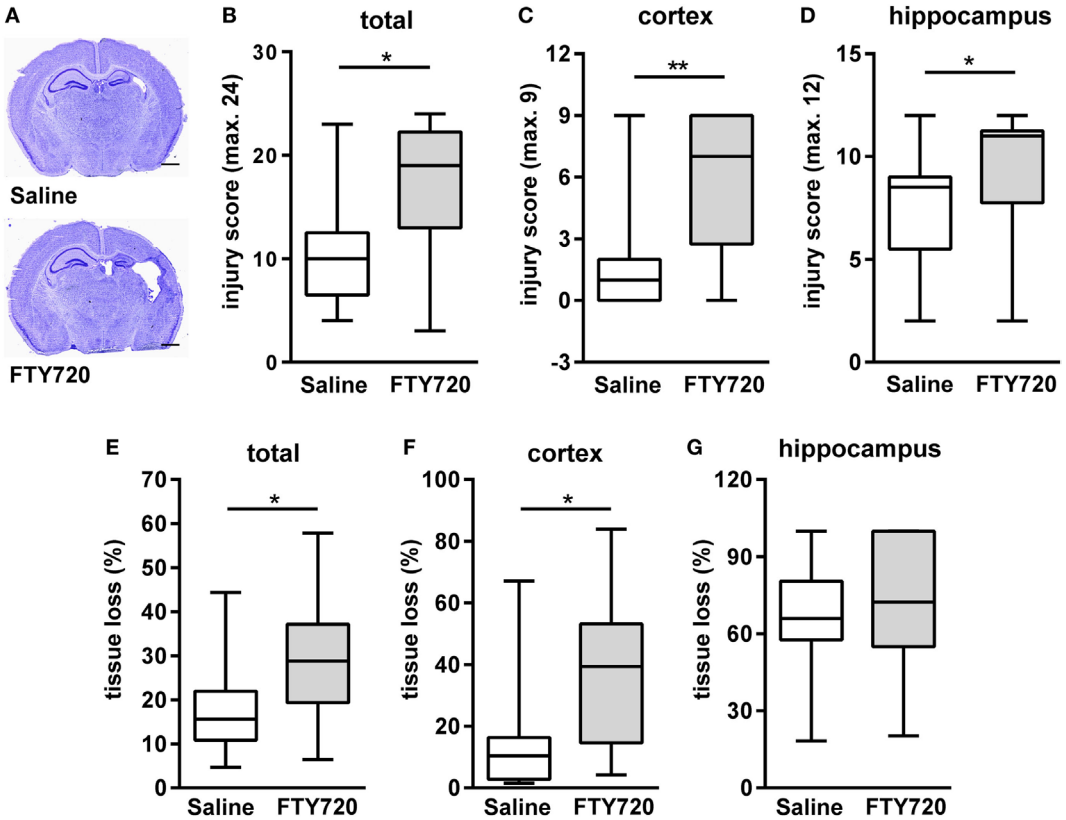

FIGURE 2 | FTY720 treatment exacerbates brain injury 1 week after neonatal HI. Histological brain injury was determined on cresyl violet stained 20 µm cryostat sections of P16 mice that were exposed to HI followed by a single intraperitoneal injection of 1 mg/kg FTY720 or 0.9% NaCl (saline) on P9. **(A)** Representative images of brain sections (−1.9 to −2.0 mm from bregma) for each experimental group are shown (scale bar: 1 mm). **(B)** Injury scores were assessed in different brain regions, e.g., cortex **(C)** and hippocampus **(D)** according to a previously described scoring system in eight regions. Each region was given a rating from 0 to 3 (0—no detectable cell loss, 1—small focal areas of neuronal cell loss, 2—columnar damage in the cortex or moderate to severe cell loss in the other regions, 3—cystic infarction and gliosis). The sum score from different regions (cortex, hippocampus, and striatum) was calculated for each animal resulting in a total maximum score of 24. **(E–G)** Brain atrophy was assessed at the level of the striatum (+0.2 to +0.3 mm from bregma) and hippocampus (−1.9 to −2.0 mm from bregma) in two consecutive sections per region and animal by measurement of intact areas in ipsilateral and contralateral hemispheres using Image J. Tissue loss was determined by comparison with contralateral values. Overall and local cortical tissue injury was significantly increased by FTY720 treatment. n = 14–16/group, $^{*}p < 0.05$, $^{**}p < 0.01$, Mann–Whitney test.

Confirming our results obtained in naïve mice (**Figure 1**), we detected a significantly reduced amount CD4 and CD8 T cells in FTY720-treated HI mice while the number of B and natural killer cells remained unchanged (**Figure 6A**). Detailed analysis of CD4 T cells revealed that Foxp3 positive regulatory T cells were similarly reduced as Foxp3 negative effector T cells by 95.5 and 98.4%, respectively (**Figure 6A**). However, we determined a significantly lower proportion of circulating regulatory T cells in the total CD4 T cell population compared to the frequency of CNS-infiltrated regulatory T cells (11.2 vs. 55.6%, $p < 0.001$; **Figures 5A** and **6A**) in saline-treated mice, confirming the selective infiltration of regulatory T cells after neonatal HI. Interestingly, the amount of circulating neutrophils and macrophages was significantly decreased in FTY720-treated mice contrasting findings in the brain (**Figure 6B**). Similarly, the proportion of inflammatory monocytes and macrophages was reduced, though not reaching significance (**Figure 6C**).

Detrimental Impact of FTY720 on Neonatal HI-Induced Brain Injury Depends on Peripheral T Cell Depletion

Whether exacerbation of ischemic brain injury in neonatal mice could be attributed to FTY720s' lymphopenic mode of action or is caused by direct neurotoxic effects was assessed in T cell depleted mice. T cell depletion was performed by anti-CD3 antibody treatment according to our previous report (26). Of note, the selected treatment protocol resulted in a strong reduction of peripheral T cells by 92% similar to that obtained after FTY720 treatment (**Figure 7A**). Neuropathological assessment and quantification of brain tissue loss revealed that antibody- and FTY720-mediated T cell depletion significantly increased HI-induced injury (**Figures 7B–D**). Of note, there was no further exacerbation of damage after FTY720 treatment in T cell-depleted mice (**Figures 7B–D**).

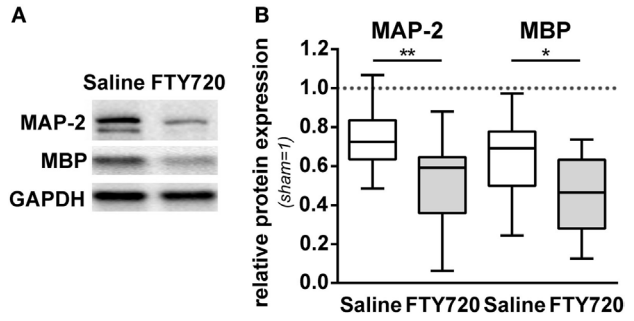

FIGURE 3 | HI-induced gray and white matter loss is increased by FTY720 treatment. P9 mice were exposed to HI followed by a single intraperitoneal injection of 1 mg/kg FTY720 or 0.9% NaCl (saline). One week after HI, protein lysates were prepared from ipsilateral hemispheres at the hippocampal level (−2.0 to −2.3 mm from bregma) from brain sections of 200 µm thickness. Following gradient sodiumdodecylsulphate electrophoreses and protein transfer, membranes were incubated with anti-myelin basic protein (MBP), anti-microtubuli associated protein-2 (MAP-2), and anti-glutaraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies **(A)**. For quantification data were normalized to the reference protein GAPDH and sham controls **(B)**. HI-induced loss of MAP-2 and MBP was significantly aggravated by FTY720 treatment. $n = 14$/group, $^{*}p < 0.05$, $^{**}p < 0.01$, unpaired *t*-test.

DISCUSSION

In contrast to adult stroke, peripheral T cell depletion by pharmacological and antibody-mediated intervention increases brain injury in a term-born equivalent model of hypoxic-ischemic brain injury. Analysis of peripheral and cerebral leukocyte subsets provided further insights into potential underlying mechanisms involving an increased infiltration of innate and particularly inflammatory cell types into the injured neonatal brain when T cells are lacking. Our unexpected results contrast findings in adult ischemia, but confirm previous reports about questionable translation from adults to neonates in experimental studies (38).

The concomitant increased infiltration of inflammatory cells, mainly neutrophils and inflammatory macrophages in the absence of T cells suggests that they have contributed to increased HI-induced brain injury. This is supported by previous work demonstrating that antibody-mediated depletion of circulating neutrophils reduces neonatal and adult ischemic brain injury (26, 39). Simultaneously reduced cell numbers of neutrophils and macrophages in the peripheral blood implicate a redistribution of inflammatory cells from the circulation to the hypoxic-ischemic brain with the total amount of infiltrated leukocytes being similar.

Expression of general endothelial adhesion molecules (i.e., VCAM and ICAM) was not modulated, suggesting that neonatal T cells regulate the migratory capacity of peripheral inflammatory cell types. So far, we can only speculate about the potential mechanisms. However, the described bias toward anti-inflammatory T cell subsets in neonates (40) might provide an endogenous protective mechanism of the neonatal organism exposed to an acute hypoxic-ischemic insult to limit excess inflammation. In the present work, we demonstrate a selective infiltration of regulatory T cells into the neonatal hypoxic-ischemic brain. Therefore, overall peripheral depletion of T cells, similarly affecting regulatory and effector T cells results in a more pronounced difference for cerebral regulatory T cell counts compared to effector T cells in HI-injured brain hemispheres. This suggests that in addition to inhibition of the migratory capacity of inflammatory cells in the periphery, the presence of regulatory T cells in the injured brain might be an important mechanism of endogenous neuroprotection. To develop optimal and specific therapies, further work is needed to specify the temporal regulation of this T cell subset and corresponding molecular mechanisms mediating neuroprotection.

In spite of these interesting findings, our results in part contradict to a previous report describing neither protection nor exacerbation of HI-induced brain injury after FTY720 treatment in postnatal day 7 rats (22). Differences in species, age, and injury severity likely provide an explanation. As such, P7 rats compared to P9 mice were used and control animals revealed up to 40% tissue loss in the cortex while in the present study on P9 mice only 10% cortical tissue loss was observed. Severity of injury and differences in experimental models are of particular importance regarding the neuroinflammatory response as already reported in adult models of ischemic stroke (41, 42). Furthermore, FTY720 was purchased from different companies potentially affecting its lymphopenic capacity. This can hardly be clarified as depletion efficiency and duration in terms of absolute T cell numbers and other leukocyte subsets were not provided (22).

To determine whether detrimental effects were caused by FTY720s' pleiotropic effects (43) independent of its lymphopenic mode of action we performed T cell depletion *via* antibody treatment which resulted in increased brain injury comparable to that induced by FTY720 single treatment. The fact that FTY720 did not further enhance HI brain injury in T cell depleted mice suggests that side effects on other neural cell types are rather unlikely. This is supported by previous work with neuronal cell cultures demonstrating no effect of FTY720 on hypoxia-induced neuronal cell death (19, 20) and by results of the present study revealing no significant changes in microglia and endothelial activation. However, we cannot completely exclude the possibility that FTY720 might have directly affected innate immune cells as previously suggested in an experimental model of adult stroke (44).

Our results of aggravated brain injury after peripheral T cell depletion seem contradicting to previous reports in neonatal rodents showing neuroprotection in $Rag1^{-/-}$ mice or by depletion of gamma delta T cells (11, 12). However, $Rag1^{-/-}$ mice lack mature T but also B cells impeding clear conclusions about the specific contribution of each immune cell subset (11). Albertsson et al. specifically deleted gamma delta T cells resulting in significant neuroprotection after neonatal HI (12). Since the proportion of this subset is rather small within the total T cell population our results suggest that the detrimental role of gamma delta T cells might have been counteracted by other more abundant protective T cell subsets in the injured brain, e.g., regulatory T cells. This is supported by our results on a selective infiltration of regulatory T cells compared to Foxp3 negative effector T cells in HI-injured brain hemispheres.

In addition to the aforementioned explanations, the most important issue to be considered when comparing these two studies is the difference in the age of animals. While Nazmi and Albertsson investigated pre-term equivalent HI models using

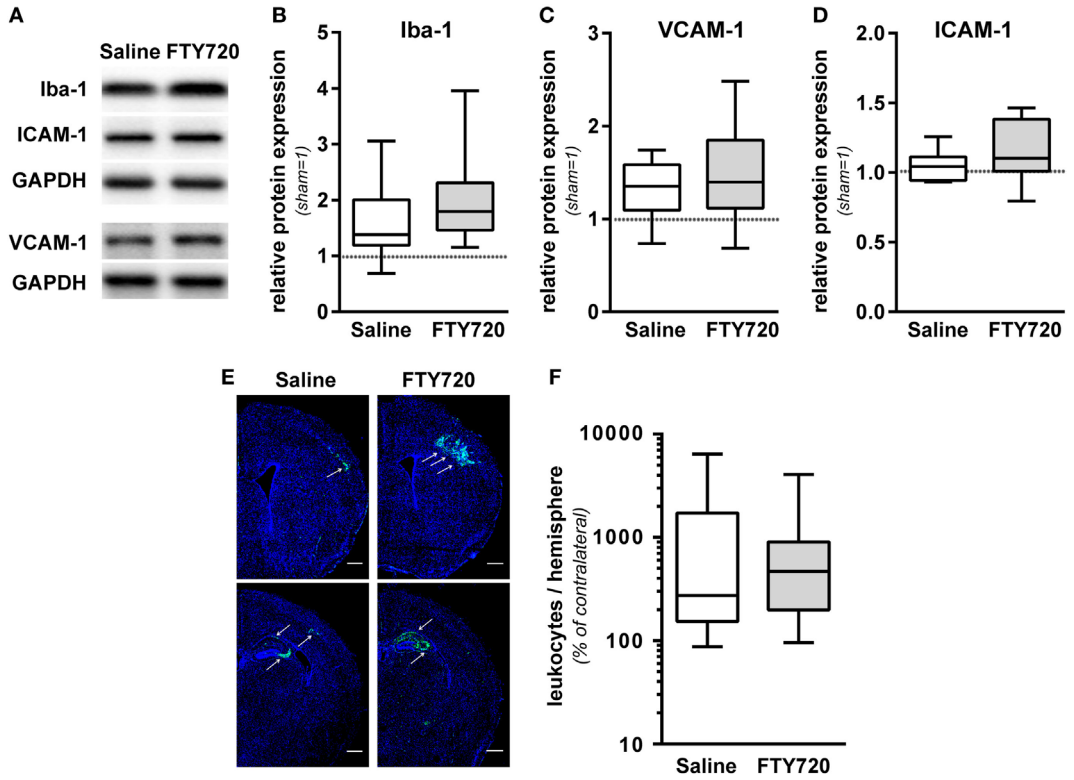

FIGURE 4 | FTY720 does not alter microglia and endothelial activation and total leukocyte infiltration. P9 mice were exposed to HI followed by a single intraperitoneal injection of 1 mg/kg FTY720 or 0.9% NaCl (saline). **(A)** On P16 microglia and endothelial activation was assessed by quantification of Iba-1 **(B)**, VCAM-1 **(C)**, and ICAM-1 **(D)** in protein lysates *via* western blot. Protein lysates were prepared from ipsilateral hemispheres at the hippocampal level (−2.0 to −2.3 mm from bregma) from brain sections of 200 µm thickness followed by gradient sodiumdodecylsulphate (SDS) electrophoresis (Iba-1 and ICAM-1) or protein separation in 12.5% SDS gels. After protein transfer, membranes were incubated with appropriate antibodies. Data were normalized to the reference protein glutaraldehyde-3-phosphate dehydrogenase (GAPDH) and sham controls. FTY720 treatment did not significantly alter Iba-1, VCAM-1, and ICAM-1 protein levels. **(E)** Leukocyte infiltration was evaluated qualitatively by immunohistochemistry for CD45 (green) 1 week after HI. Nuclei were counterstained with Dapi (blue). Representative images show maximal intensity projections of confocal z-stacks at the level of striatum and at the level of the hippocampus (scale bar: 500 µm). Leukocyte infiltration was most prominent in regions mainly affected by the detrimental effect of FTY720, e.g., the hippocampus and cortex (indicated by arrows) **(E)**. Quantification of leukocyte infiltration was performed by flow cytometry after isolation of single cell suspensions and myelin removal *via* Percoll gradient centrifugation **(F)**. Absolute cell counts of viable $CD45^{high}$ cells were determined in contralateral and ipsilateral hemispheres using TrueCount beads. Numbers in ipsilateral hemispheres were related to contralateral hemispheres of the same animals to correct for inter-experimental variations due to isolation procedures. Two hemispheres were pooled per sample. Total HI-induced peripheral leukocyte infiltration was not significantly changed by FTY720. n = 14/group for **(A–D)**, n = 30–31 samples/group [pooled analysis from measurements for panel 1/lymphoid, panel 2/regulatory T cells (**Figure 5A**), and panel 3/myeloid (**Figure 5B**)] for **(E)**.

P4 and P5 mice we used a term-born equivalent injury model. This may strongly modulate the impact of immunomodulatory interventions because the immune system and the brain are still developing. As such, white matter development and axonal outgrowth in the rodent CNS between P1 and P7 correspond to 23–36 weeks gestation in humans (45). Therefore, rodents at P9–10 are meanwhile considered to be more comparable to term infants regarding brain development (46, 47). According to that, infiltrated T cells might interfere with vulnerable CNS maturation processes taking place between p4–p7 (48), while in the term-born equivalent brain of P9–P10 rodents T cells may rather protect from HI-induced destruction of already differentiated and matured neural cells, a hypothesis that needs to be proven in future studies. In addition, changes in the immune system between P5 and P9 might explain differences in outcome. Even though comprehensive ontogenetic investigations of different immune cell subsets in neonatal mice are sparse, few reports described pronounced changes in the immune system. These involve, for example, a continuous increase in the proportion of lymphocytes while neutrophils decrease within the first 2 weeks of life (49, 50). A detailed characterization of T cell phenotypes over time is still missing.

Instead of $Rag1^{-/-}$ mice, we used an antibody-mediated cell depletion approach, which might not be as efficient as genetic ablation. However, a strong reduction of circulating T cells by 92% was achieved in the present work. To dissect the specific role of T cells in $Rag1^{-/-}$ mice adoptive transfer of B cells is needed, which is technically challenging in neonatal mice *via* the intravenous route. Studies in adult brain ischemia revealed similar outcomes with regard to histological brain injury in $Rag1^{-/-}$ reconstituted with B cells compared to anti-CD3 treatment (26, 33) suggesting that antibody-mediated depletion is an appropriate approach. In order to mimic FTY720-induced reduction in peripheral T cells, we performed depletion throughout the

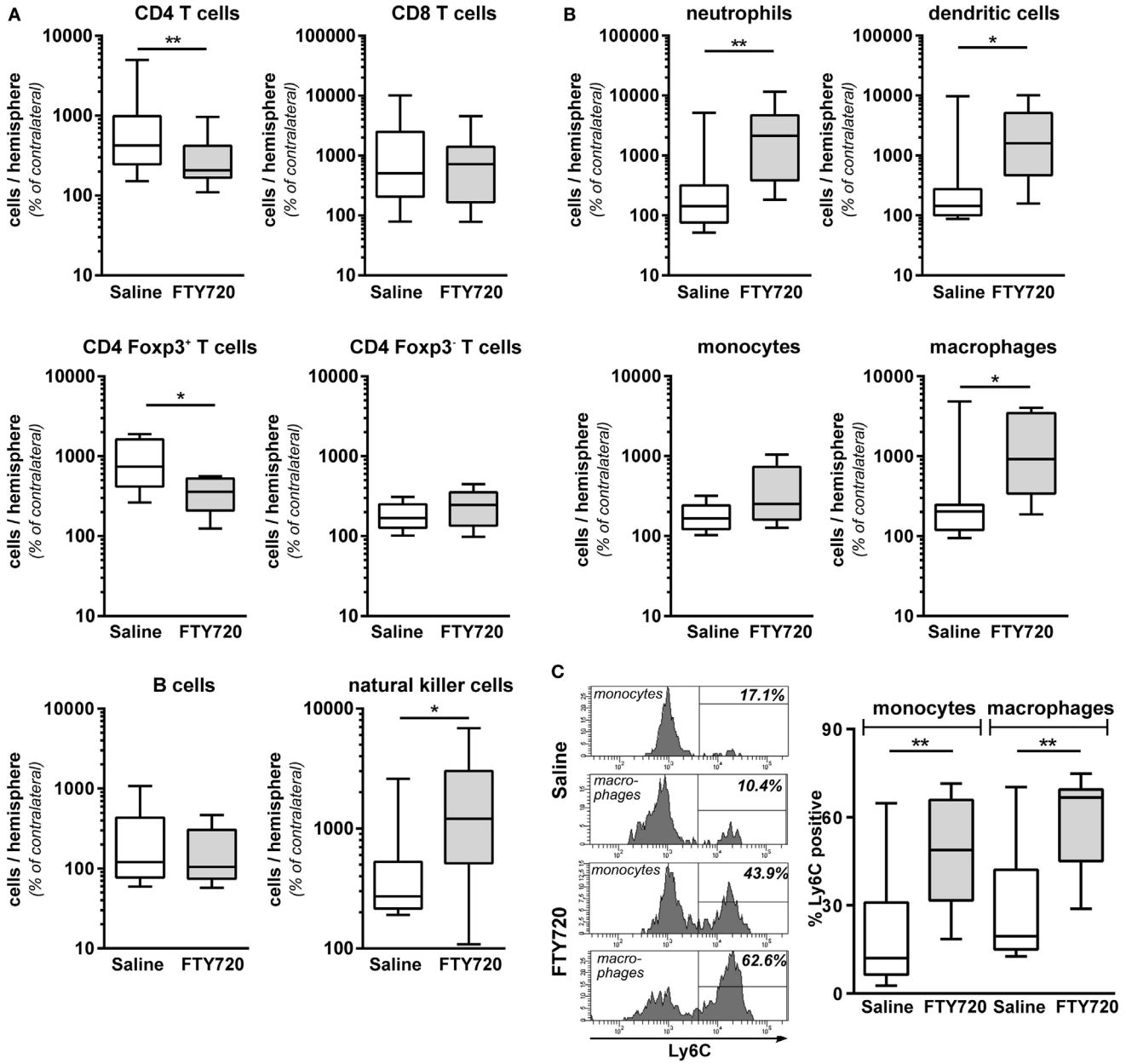

FIGURE 5 | Peripheral T cell depletion by FT720 reduces the amount of T cells but increases innate immune cells in the hypoxic-ischemic brain. Multichannel flow cytometry was performed on immune cells isolated from ipsilateral and contralateral hemispheres obtained from P16 mice that were exposed to HI followed by a single intraperitoneal injection of 1 mg/kg FTY720 or 0.9% NaCl (saline) on P9. Different immune cell subsets were identified according to the gating strategy provided in Figure S1 in Supplementary Material. Briefly, viable peripheral leukocytes were identified as $CD45^{high}$FVD (fixable viability dye)$^-$ cells and further subdivided into B lymphocytes ($CD19^+$), natural killer cells ($NK1.1^+$), CD4 T cells ($CD19^-$, $NK1.1^-$, $CD3^+$, $CD4^+$), CD8 T cells ($CD19^-$, $NK1.1^-$, $CD3^+$, $CD8^+$), and regulatory T cells ($CD3^+$, $CD4^+$, $Foxp3^+$) T cells **(A)**. For analysis of myeloid subsets viable leukocytes were gated for lymphocyte-depleted cells ($CD3^-$, $NK1.1^-$, $B220^-$) and further subdivided into monocytes ($CD115^+$, $Ly6G^-$) and neutrophils ($CD115^-$, $Ly6G^+$). Remaining cells were gated by CD11b and CD11c to distinguish dendritic cells ($Ly6G^-$, $CD115^-$, $CD11c^{high}$) and macrophages ($Ly6G^-$, $CD115^-$, $CD11c^-$, $CD11b^+$) **(B)**. Absolute lymphocyte **(A)** and myeloid **(B)** cell counts were quantified in contralateral and ipsilateral hemispheres. Numbers in ipsilateral hemispheres were related to contralateral hemispheres of the same animals to correct for inter-experimental variations due to isolation procedures. For monocytes and macrophages, the proportion of inflammatory cells was quantified based on their Ly6C expression **(C)**. FTY720 treatment significantly reduces the amount of infiltrated CD4 T cells and particularly regulatory T cells, which was accompanied by an increased infiltration of innate especially inflammatory myeloid cell types. n = 8–22 samples/group (two hemispheres pooled per sample), $^*p < 0.05$, $^{**}p < 0.01$, unpaired t-test for $Foxp3^+$ and $Foxp3^-$ CD4 T cells in **(A)** and monocytes in **(B)**, remaining: Mann–Whitney test.

entire observation period. Considering the suggested biphasic T cell infiltration pattern (7), we cannot determine the relevance of these two infiltration peaks separately. More mechanistic analyses with time-specific deletion will be needed to clarify the pathophysiological role of T cell infiltration at early and later time points.

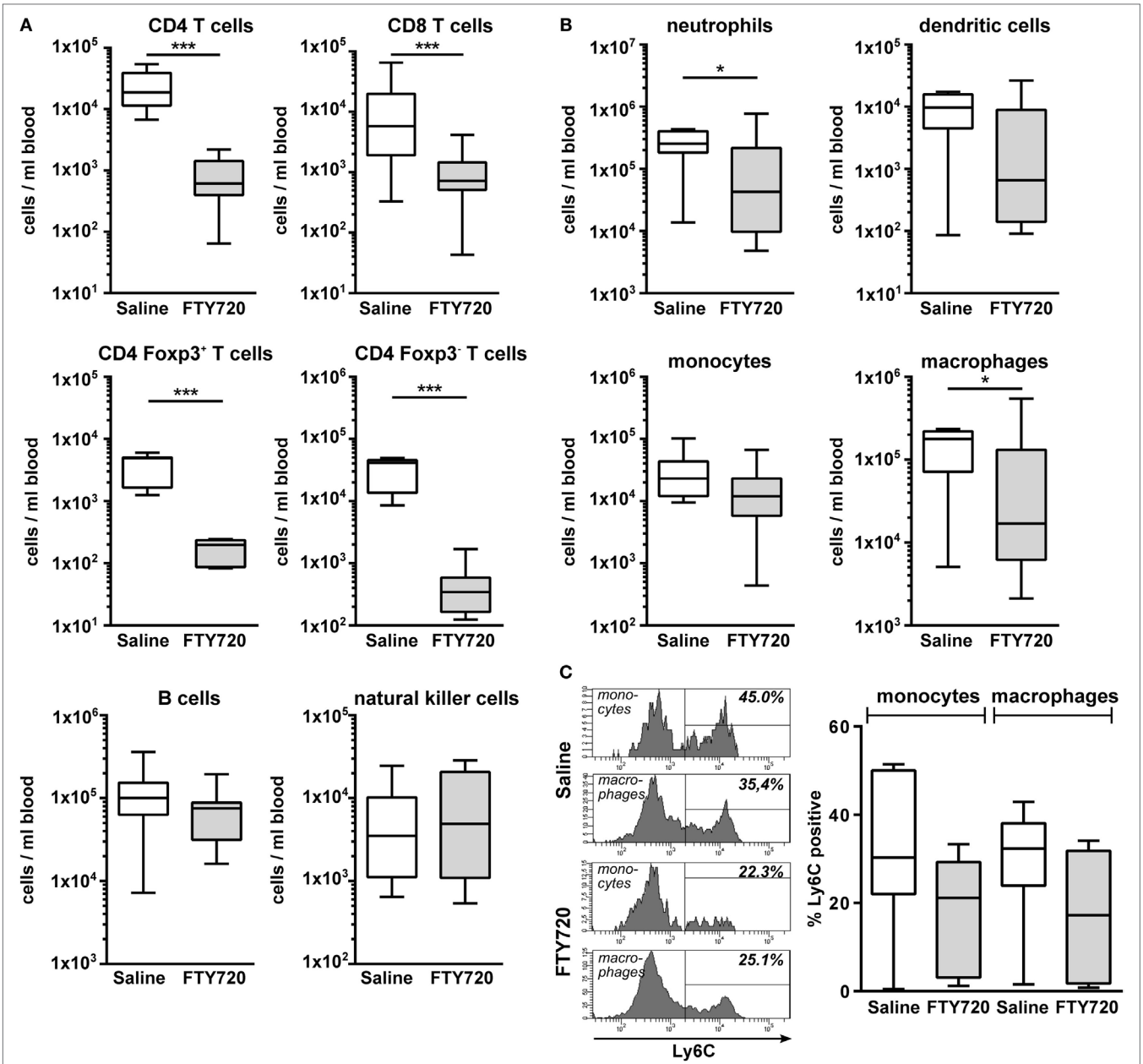

FIGURE 6 | FTY720 treatment results in reduced peripheral T cell, neutrophil, and macrophage counts in neonatal HI-injured mice. Immune cell subset analysis in the peripheral blood was performed in P16 mice that were exposed to HI followed by a single intraperitoneal injection of 1 mg/kg FTY720 or 0.9% NaCl (saline) on P9. Different immune cell subsets were identified according to the gating strategy provided in Figure S1 in Supplementary Material. Briefly, viable peripheral leukocytes were identified as CD45$^+$FVD (fixable viability dye)$^-$ cells and further subdivided into B lymphocytes (CD19$^+$), natural killer cells (NK1.1$^+$), CD4 T cells (CD19$^-$, NK1.1$^-$, CD3$^+$, CD4$^+$), CD8 T cells (CD19$^-$, NK1.1$^-$, CD3$^+$, CD8$^+$), and regulatory T cells (CD3$^+$, CD4$^+$, Foxp3$^+$) T cells **(A)**. For analysis of myeloid subsets viable leukocytes were gated for lymphocyte-depleted cells (CD3$^-$, NK1.1$^-$, B220$^-$) and further subdivided into monocytes (CD115$^+$, Ly6G$^-$) and neutrophils (CD115$^-$, Ly6G$^+$). Remaining cells were gated by CD11b and CD11c to distinguish dendritic cells (Ly6G$^-$, CD115$^-$, CD11c^{high}) and macrophages (Ly6G$^-$, CD115$^-$, CD11c$^-$, CD11b$^+$) **(B)**. Absolute lymphocyte **(A)** and myeloid **(B)** cell counts were quantified. For monocytes and macrophages, the proportion of inflammatory cells was quantified based on their Ly6C expression **(C)**. The amount of circulating T cells and of neutrophils and macrophages is significantly reduced in FTY720-treated HI mice, while the number of B, natural killer, and dendritic cells remained unchanged. n = 7–19/group unpaired t-test for Foxp3$^+$ and Foxp3$^-$CD4 T cells in **(A)** and Ly6C$^+$ cells in **(C)**, remaining: Mann–Whitney test.

In conclusion, the present work demonstrates that in contrast to adult stroke. FTY720 worsens neonatal HI-induced brain injury, most likely through sustained depletion of peripheral T cells. These results highlight that caution is needed when transferring findings from adult animal models to the developing brain. The time point of injury and intervention seems to be critical for injury outcome after immunomodulatory interventions. Furthermore, the current results suggest that neonatal T cells promote endogenous neuroprotection

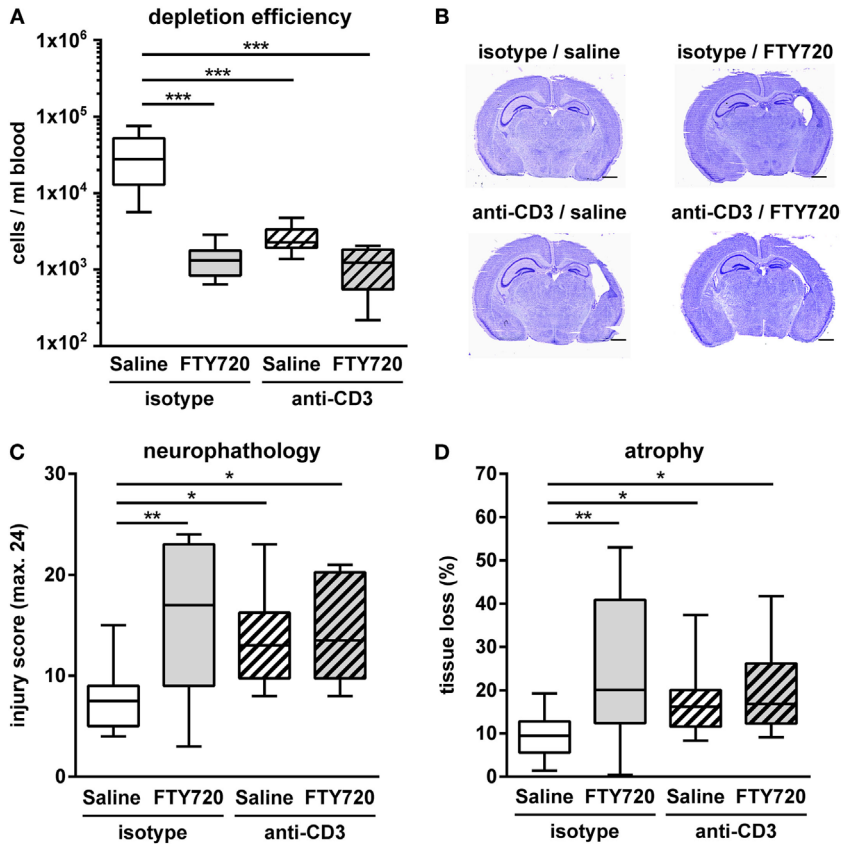

FIGURE 7 | Exacerbation of HI-induced brain injury by FTY720 is dependent on peripheral T cells. Whether FTY720s' detrimental effect could be attributed to its T cell depleting effect was determined in anti-CD3-treated animals. P9 mice were exposed to HI followed by a single intraperitoneal injection of 1 mg/kg FTY720 or 0.9% NaCl (saline). Anti-CD3 treatment was started on P8 followed by repetitive injections every 48 h according to our previous report (26). Control mice received an isotype control antibody. One week after HI, depletion efficiency by antibody- and FTY720-treatment as well as in the combined setting was analyzed in the peripheral blood *via* flow cytometry **(A)**. Brain injury was evaluated on cresyl violet stained tissue sections on P16 **(B,C)**. Neuropathological assessment was performed according to a previously described scoring system in eight regions. Each region was given a rating from 0 to 3 (0—no detectable cell loss, 1—small focal areas of neuronal cell loss, 2—columnar damage in the cortex or moderate to severe cell loss in the other regions, 3—cystic infarction and gliosis). The sum score from different regions (cortex, hippocampus, and striatum) was calculated for each animal resulting in a total maximum score of 24 **(C)**. Brain tissue atrophy was determined by measurement of intact areas in ipsilateral and contralateral hemispheres using Image J. Tissue loss was determined by comparison with contralateral values **(D)**. Anti-CD3 and FTY720-mediated T cell depletion significantly increased HI-induced injury. The combined treatment resulted in a similar degree of injury compared to single treatments. n = 8–10/group for **(A)**, n = 10–14/group for **(B–D)**, one-way ANOVA with Bonferroni *post hoc* test for **(A,C)**, Kruskal–Wallis with Dunn's multiple comparison test for **(D)**.

with regulatory T cells of particular importance in this context. Further in depth analysis specifically targeting this T cell subset may provide new opportunities for therapeutic interventions.

ETHICS STATEMENT

Experiments were performed in accordance to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines with government approval by the State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia.

AUTHOR CONTRIBUTIONS

JH, CK, HA, MC, and IB performed experiments and analyzed the data. JH and IB initiated, designed, and organized the study. JH, IB, WH, and UF-M wrote the manuscript.

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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.2018.01696/full#supplementary-material.

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Excitability of Rat Superficial Dorsal Horn Neurons Following a Neonatal Immune Challenge

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Previous studies have shown that neonatal exposure to a mild inflammatory challenge, such as lipopolysaccharide (LPS, *Salmonella enteriditis*) results in altered pain behaviors later in life. To further characterize the impact of a neonatal immune challenge on pain processing, we examined the excitability of superficial dorsal horn (SDH) neurons following neonatal LPS exposure and subsequent responses to noxious stimulation at three time-points during early postnatal development. Wistar rats were injected with LPS (0.05 mg/kg i.p.) or saline on postnatal days (PNDs) 3 and 5, and later subjected to the formalin test at PNDs 7, 13, and 22. One hour after formalin injection into the plantar hindpaw, animals were euthanized (Ketamine, 100 mg/kg i.p.) and transverse slices from the lumbosacral spinal cord were prepared. Whole-cell patch-clamp recordings were made from SDH neurons (KCH_3SO_4-based internal, 22–24°C) on the ipsi- and contralateral sides of the spinal cord. Depolarising current steps were injected into SDH neurons to categorize action potential (AP) discharge. In both saline- and LPS-treated rats we observed age-related increases the percentage of neurons exhibiting tonic-firing, with concurrent decreases in single-spiking, between PND 7 and 22. In contrast, neonatal exposure to LPS failed to alter the proportions of AP discharge patterns at any age examined. We also assessed the subthreshold currents that determine AP discharge in SDH neurons. The rapid outward potassium current, I_{Ar} decreased in prevalence with age, but was susceptible to neonatal LPS exposure. Peak I_{Ar} current amplitude was greater in ipsilateral vs. contralateral SDH neurons from LPS-treated rats. Spontaneous excitatory synaptic currents (sEPSCs) were recorded to assess network excitability. Age-related increases were observed in sEPSC frequency and time course, but not peak amplitude, in both saline- and LPS-treated rats. Furthermore, sEPSC frequency was higher in ipsilateral vs. contralateral SDH neurons in LPS-treated animals. Taken together, these data suggest a neonatal immune challenge does not markedly affect the intrinsic properties of SDH neurons, however, it can increase the excitability of local spinal cord networks via altering the properties of rapid A-type currents and excitatory synaptic connections. These changes, made in neurons within spinal cord pain circuits, have the capacity to alter nociceptive signaling in the ascending pain pathway.

Keywords: action potential, LPS, pain, potassium current, EPSC

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INTRODUCTION

The long-term consequences of early life events, such as neonatal stress and infection, are well documented. For example, rats exposed to the bacterial mimetic, lipopolysaccharide (LPS), during the first postnatal week exhibit potentiated stress responses when tested as adults. This has been attributed to permanent alterations within the hypothalamic-pituitary-adrenal (HPA) axis (1). One commonly used method to reliably and rapidly activate the HPA axis is the formalin test. This test, initially described by Dubuisson and Dennis (2), involves formalin injection into the hindpaw. It causes an inflammatory response and characteristic bi-phasic pain-induced behaviors (flinching and licking). Our group has shown previously that rats exposed to LPS at postnatal days (PNDs) 3 and 5 exhibit increased formalin-induced behavioral responses during both pre-adolescence (PND 13; (3) and adulthood (PND 80–97; (4). These findings demonstrate that neonatal exposure to LPS can have long-lasting effects on pain behavior. Surprisingly, limited data exists on the spinal cord neurons and mechanisms that underlie these long-lasting changes in pain processing.

The superficial dorsal horn (SDH) of the spinal cord is the first processing node in the ascending pain pathway. We have known for more than two decades that peripheral immune challenges can increase the concentration of pro-inflammatory substances within the adult spinal cord (5). Moreover, bath application of pro-inflammatory substances to spinal cord slices can alter the properties of SDH neurons. These alterations include decreases in input resistance in response to prostaglandin E2 (6) and increased excitatory drive following exposure to BDNF (7). In addition, formalin injection in adult animals can increase spinal cord metabolic activity (8) and alter dorsal horn signaling (9, 10). Thus, it is clear that an early immune challenge causes changes in the adult spinal cord, however, little is known about the nature of these changes or when they occur during the early postnatal period. This is important as plasticity within the rodent spinal cord occurs during critical periods during postnatal development (11).

In order to understand the neuronal mechanisms underlying the long-term changes in adult pain behaviors after neonatal insults, several studies have investigated SDH neuron properties in response to neonatal injuries. Injection of inflammatory agents into the hindpaw of neonatal rats results in spreading of the central arbors of primary afferent fibers, 8–10 weeks later (12). This has implications for overall network excitability. Indeed, when examining network excitability following a surgical incision of the mid-thigh, Li, and colleagues (13) observed an increase in the frequency of excitatory postsynaptic currents (EPSCs) within SDH neurons. Further studies utilizing a similar neonatal surgical incision also revealed alterations in the intrinsic properties of individual SDH neurons in the adult (14). Together, these studies indicate that the SDH contains a neuronal population that is highly plastic during the early postnatal period and susceptible to perturbations after peripheral inflammation and/or injury.

Despite the evidence that neonatal injury, immune challenge and formalin injection can alter SDH properties in adult rats, limited information exists on the effect of neonatal exposure to LPS and subsequent formalin injection on the developmental trajectory of SDH neurons. Previously, our group has examined the intrinsic properties of SDH neurons and observed altered intrinsic properties of LPS-challenged rats at PND 22 formalin challenge (3). Based on these findings we proposed that a neonatal exposure to LPS can alter the excitability of SDH neurons long after the initial immune response has passed. Therefore, in order to build upon these previous initial findings, the present study reports whole-cell responses from SDH neurons following formalin injection in rats previously challenged with a neonatal exposure to LPS or saline. We demonstrate significant developmental changes in rat SDH neurons and an increase in overall network excitability within the SDH following neonatal inflammation. These data provide insight into the mechanisms underlying neuron plasticity at the first node in the ascending pain pathway during a noxious event following an early life immune challenge.

MATERIALS AND METHODS

Experimental Procedures

All protocols were approved by the University of Newcastle Animal Care and Ethics Committee, and were undertaken in accordance with the 2004 National Health and Medical Research Council of Australia Code of Practice covering the use of animals for scientific purposes. Eight naïve female Wistar rats were obtained from the University of Newcastle Animal House and allowed 1-week acclimatization prior to mating in a vivarium. Mating resulted in 86 offspring, and 58 were used in this study. After birth, at PNDs 3 and 5, the rat pups were briefly removed from their home boxes, weighed and given an IP injection of either LPS (*Salmonella enterica,* serotype *enteriditis*; Sigma Aldrich chemical Co., USA, dissolved in sterile pyrogen-free saline, 0.05 mg/kg) or an equal volume of saline (Livingstone International, Australia). We have previously confirmed that this concentration of LPS raises the circulating levels of cytokines (IL-1β) and plasma corticosterone (3), as well as the levels of pro-inflammatory cytokines IL-1β and TNF-α in the hippocampus (15). The pups were then left undisturbed with their dams in a temperature- (21 ± 1°C) and humidity- (60%) controlled environment, under a 12/12 h light/dark cycle with food and water available *ad libitum*. All pups from the same litter were treated identically.

Formalin Behavioral Testing and Analysis

Rats were randomly assigned to three age groups (PND 7, 13, and 22) with a maximum of three pups per litter being assigned to each group. On the test-day, each rat received a 10 μl injection of formalin into the plantar surface of the hindpaw via a 31 gauge needle; PND 7 rats were injected with 0.5% formalin, PND 13 with 0.8% formalin, and PND 22 with 1.1% formalin. These formalin concentrations were used because previous studies demonstrated they are sufficient to induce the characteristic bi-phasic behavioral response at each age (16, 17). The test apparatus and conditions have been described elsewhere in detail by our group (17). We did not examine any responses to injection of saline into the hindpaw as it has been previously shown that rats

subjected to such an injection do not display flinching or licking behaviors when tested at any developmental stage (16, 18, 19).

Behavioral responses to the formalin injection were scored according to the method of Wheeler-Aceto and Cowan (20). Testing in the hour following formalin injection was divided into an early (the first 5 min) and late phase (10–60 min) during which flinching frequency and licking duration of the injected paw (in seconds) were scored. A detailed description of the statistical analysis and resulting data can be found in a previous publication from our group (3). Briefly, whilst no differences in behavioral responses were observed at PND 7, an increase in licking was observed at PND 13 and an increase in flinching behaviors at PND 22.

Electrophysiological Recordings

Following the formalin test, spinal cords from 58 rats (PND 7 = 15; PND 13 = 18; PND 22 = 25; equally distributed between treatment groups and sexes) were dissected for subsequent electrophysiological recordings from superficial dorsal horn (SDH, laminae I, and II) neurons. For this procedure the rats were anesthetized with Ketamine (100 mg/kg) and decapitated. The vertebral column and posterior thoracic wall were isolated and rapidly immersed in ice cold sucrose substituted artificial cerebrospinal fluid (sACSF) containing (in mM): 250 sucrose, 25 $NaHCO_3$, 10 glucose, 2.5 KCl, 1 NaH_2PO_4, 1 $MgCl_2$, and 2.5 $CaCl_2$, continuously bubbled with 95% O_2- 5% CO_2 (pH of 7.3–7.4; (21). The lumbosacral enlargement of the spinal cord was then removed and marked to allow identification of the ipsi- and contralateral dorsal horns - relative to the injection site. Transverse slices from L3-5 spinal segments (300 μm thick) were prepared using a vibratome (Leica VT1200s, Leica Microsystems, Wetzlar, Germany). Slices were transferred to an interface storage chamber containing ACSF (118 mM NaCl substituted for sucrose in sACSF) and allowed to equilibrate for 1 h at room temperature (22–24°C) before recording commenced.

Slices were transferred to a recording chamber (volume 0.4 ml) and continually superfused with ACSF (4–6 bath volumes/minute). Recording temperature was maintained at 22–24°C via an in-line temperature control unit (TC-324B, Warner Instruments, Hamden, CT). Whole-cell patch-clamp recordings were obtained from SDH neurons visualized with infrared differential contrast optics and an infrared-sensitive camera (Rolera-XR, Olympus, NJ). Patch pipettes (3–4 MΩ resistance) were prepared from thin walled borosilicate glass (PG150T-15, Harvard Apparatus, Kent UK) and filled with a potassium-based internal containing (in mM): 135 KCH_3SO_4, 6 NaCl, 2 $MgCl_2$, 10 HEPES, 0.1 EGTA, 2 MgATP, 0.3 NaGTP, pH 7.3 (with 1 M KOH). Whole-cell patch-clamp recordings were obtained using a Multiclamp 700B Amplifier (Molecular Devices, Sunnyvale, CA). The whole-cell recording configuration was first established in voltage clamp mode (holding potential −60 mV). Series resistance was measured from the averaged response (five trials) to a hyperpolarizing pulse (5 mV amplitude, 10 ms duration). This was measured at the beginning and end of each recording session and data were rejected if values changed by >20%. While in the voltage clamp recording mode, the presence of the major subthreshold currents, known to be present in rodent SDH neurons (21, 22), was assessed by delivering a hyperpolarizing pulse (to −90 mV, 1 s duration) immediately followed by a depolarizing step (to −40 mV, 200 ms duration). This was repeated five times to obtain an average for analysis. In addition to the above protocol, spontaneous excitatory postsynaptic events (sEPSCs) were recorded in voltage clamp over a period of 60–120 s from a holding potential of −70 mV.

Following the completion of the above voltage clamp protocols, the amplifier was switched to current clamp mode. The membrane potential observed ∼15 s after this switch was taken as resting membrane potential (RMP). All current clamp recordings were made from this potential and reported membrane potentials have been corrected for a 10 mV liquid junction potential (23). Individual action potential (AP) properties and discharge categories were examined by injecting a series of depolarizing and hyperpolarizing current steps (20 pA increments, 800 ms duration, delivered every 8 s).

Data Capture and Analysis

Data were digitalized online (sampled at 20 kHz; sEPSCs filtered at 2 kHz, all other data filtered at 6 kHz) via an ITC-16 computer interface (Instrutech, Long Island, NY) and stored on a Macintosh computer using Axograph X software (Molecular Devices). Data were analyzed offline using Axograph X software. sEPSCs were detected and captured using the scaled template method (24, 25). Upon inspection, events were rejected if they overlapped or did not include a stable baseline prior to the rising phase of the captured event. Several sEPSC parameters were measured, including peak amplitude and rise and decay times as well as the charge transfer (26). Because the rapid A-type potassium current (I_{Ar}) was the dominant current observed at all ages in both saline- and LPS-treated rats, its features were analyzed further as done previously (21). I_{Ar} amplitude was measured by subtracting the amplitude of any steady-state current (in the last 50 ms of the depolarizing step) from the maximal I_{Ar} current peak. The decay phase of the I_{Ar} response was fit with a single exponential (over 20–80% of its falling phase). Individual APs were captured using the derivative threshold method, using the optimum threshold for each age group examined (PND 7, dv/dt = 10; PND 13, dv/dt = 15; PND 22, dv/dt = 20). AP threshold was measured at the inflection point during the rising phase of the AP. Rheobase current was defined as the smallest step-current that elicited at least one AP. The amplitude of each AP was measured as the difference between its threshold and maximum positive peak. AP half-width was calculated at 50% of AP amplitude. AP afterhyperpolarization was measured as the difference between AP threshold and its maximum negative peak.

Statistics

Comparisons were made in one of two ways: (1) across postnatal developmental, by comparing data for contralateral SDH neurons at each age within each treatment group, or (2) to assess the impact of neonatal exposure to LPS upon a subsequent injection of formalin, by comparing treatment groups within each age group using SPSS v25. G tests, with Williams'

correction, were used to determine whether the proportions of discharge categories, responses to hyperpolarizing current and subthreshold currents differed between either ages or treatment groups. Properties of the rapid A current and sEPSC parameters were analyzed by three-way ANOVAs with Tukey *post-hoc* tests. Data that failed Levene's test for homogeneity of variance were compared using the non-parametric Mann-Whitney test. Statistical significance was set at $p < 0.05$ and all data are presented as means ± SEM.

RESULTS

A total of 228 recordings (PND 7 = 65; PND 13 = 77; PND 22 = 86) were obtained from SDH neurons in 58 rats (PND 7 = 15; PND 13 = 18; PND 22 = 25; equally distributed between treatment groups and sexes). Recordings were made from neurons classified as either ipsi- or contralateral according to injection side (i.e., left or right hindpaw), and sample numbers reflect those from previous studies examining SDH neuronal properties (11, 21). Recording conditions, as assessed by series resistance and holding current, did not differ between any of the groups examined in this study. Data were compared in two ways: (1) during postnatal development—by comparing contralateral SDH neuron responses across the three ages examined, or (2) to assess the impact of neonatal exposure to LPS upon a subsequent injection of formalin—by comparing ipsi- or contralateral SDH neuron responses from saline- or LPS-treated rats within each age group. In our previous study, we reported the intrinsic properties of these neurons, including input resistance, resting membrane potential, rheobase and AP properties (3). Previously, we reported remarkably similar intrinsic properties at PND 7 and 13, with a decrease in input resistance and AP amplitude in ipsilateral SDH neurons from LPS-treated animals at PND 22. These previous data underpin our current hypothesis that a neonatal exposure to LPS alters the excitability of SDH neurons long after the initial immune response has passed. To extend our previous findings, in the current study we provide a detailed account of the properties that shape the excitability of these SDH neurons within dorsal horn circuits. These properties include the excitability of individual SDH neurons as measured by injection of current steps, the whole-cell currents underlying neuronal excitability, and the excitability of the SDH network by measuring the spontaneous excitatory post-synaptic currents (sEPSCs).

Action Potential Discharge

SDH neurons displayed one of five classic AP discharge patterns, at resting membrane potential, in response to depolarizing current step injection (**Figure 1**, upper panel). Tonic firing neurons discharged APs for the entire duration of the current injection; initial bursting neurons discharged a brief burst of APs at the onset of current injection; delayed firing neurons discharged APs after a delay from current onset; single spiking neurons discharged only a single AP at the onset of current injection regardless of current step magnitude; and reluctant firing neurons did not discharge any APs in response to depolarizing current injection. Reluctant firing neurons were included in this dataset as they had similar input resistance and RMP to the other categories but were capable of AP discharge under alternative recording conditions, such as when membrane potential was altered (27, 28).

We observed age-related changes in the proportions of AP discharge categories. These changes were observed in both saline- and LPS-treated rats (G *statistic* = 10.14, $p = 0.04$ and G *statistic* = 12.01, $p = 0.02$, respectively). The proportions of tonic firing neurons increased, whereas that of single spiking neurons decreased (**Figure 1**, lower panels). In contrast, there were no differences in the proportions of the various AP discharge categories between saline- and LPS- treated animals at any of the three ages examined. There were also no differences in the prevalence of the AP discharge categories in neurons from the ipsi- and contralateral sides of the spinal cord. Taken together, these data show rat SDH neurons are still plastic (i.e., altering their properties or maturing) during the first 3 weeks of postnatal development, however, the development of AP discharge categories is unaffected by neonatal exposure to LPS.

Responses to Hyperpolarizing Current Injection

Injection of hyperpolarizing current revealed one of five responses in SDH neurons (**Figure 2**, upper panels). Neurons displaying a passive response are characterized by the absence of active conductances during or after the hyperpolarizing current injection. In some neurons, we observed a "sag" in the voltage trace during the hyperpolarization step, which has been associated with the hyperpolarization-activated mixed cationic inward current [I_H; (29)]. In the remaining neurons, we observed three types of responses upon release from hyperpolarization: an extended hyperpolarization where it took some time (>200 ms) for membrane potential to return to RMP; and a rebound depolarization which occurred with or without AP discharge. These responses upon release from hyperpolarization have been previously associated with calcium subthreshold currents (30).

In all groups, passive responses to hyperpolarizing current injection dominated (**Figure 2**, lower panels). There were no significant age-related changes in the prevalence of the five responses to hyperpolarizing current injection in either saline- or LPS-treated rats (G *statistic* = 5.92, $p = 0.31$ and G *statistic* = 3.39, $p = 0.64$, respectively). Furthermore, at each age examined, neurons from saline- and LPS-treated rats displayed similar proportions of the responses to hyperpolarizing current injection. There were also no differences between the prevalence of neurons displaying each of the responses to hyperpolarizing current from the ipsi- and contralateral sides of the SDH. As with the AP discharge categories above, it appears that neonatal exposure to LPS does not affect the developmental trajectory of SDH neurons responses to hyperpolarizing current.

Subthreshold Currents

We next examined the presence and proportions of subthreshold currents known to underlie the above responses to depolarizing and hyperpolarizing current injection in a subset of SDH neurons (PND 7 = 59; PND 13 = 59; PND 22 = 66). The subthreshold current could not be determined in a subset of these neurons

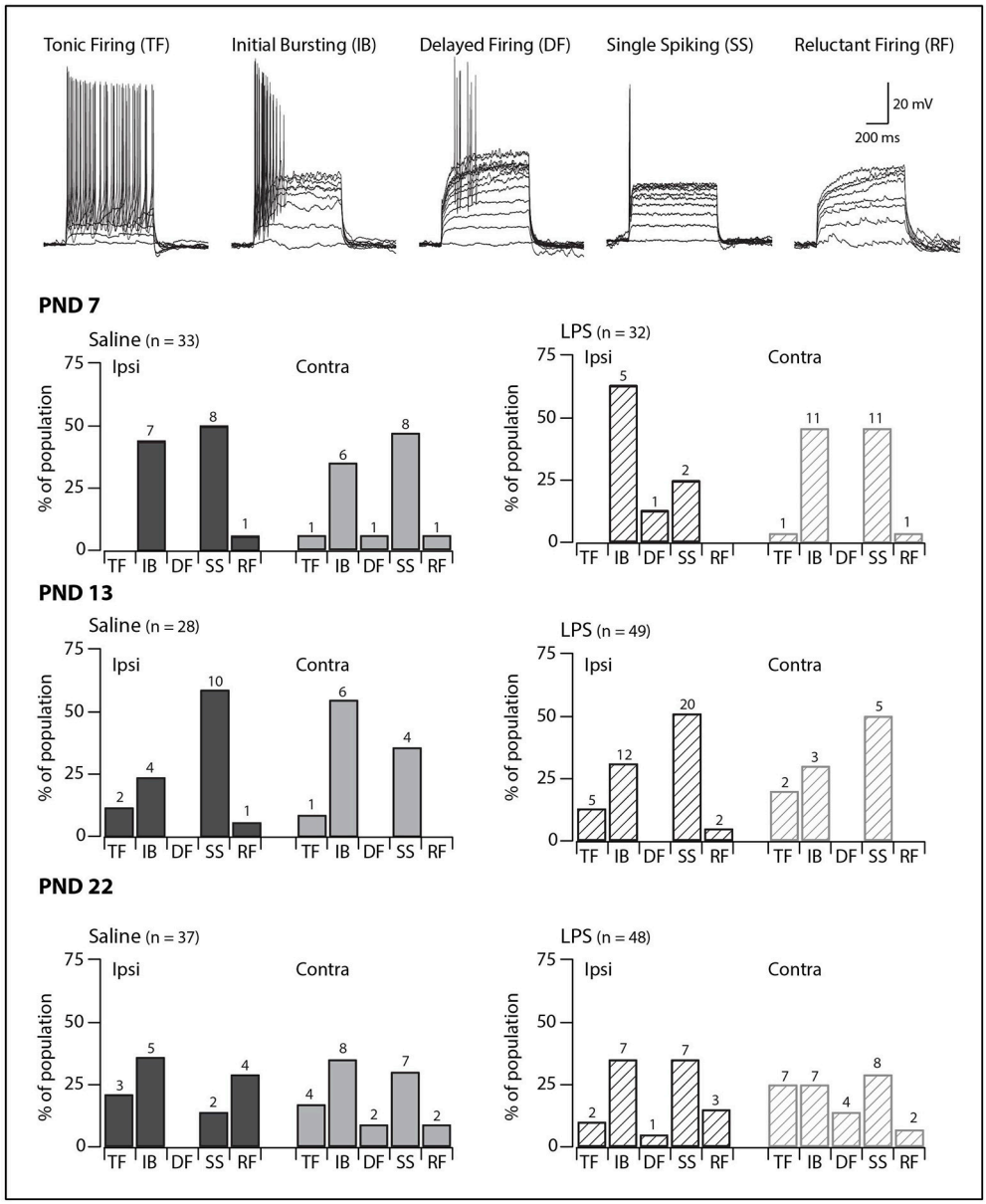

FIGURE 1 | Prevalence of AP discharge categories. Upper traces: representative traces displaying the five types of AP discharge observed in SDH neurons. All traces are from saline-treated PND 22 SDH neurons (holding potential −60 mV). Lower bar plots: demonstrate the prevalence of AP discharge categories at the ages examined (PND 7, 13 and 22, *n* values for each bar indicated). SDH neurons were recorded both ipsilateral (dark gray) and contralateral (light gray) to the formalin injection side in animals pre-treated with either saline (solid bars) or LPS (hashed bars) when they were neonates. In both treatment groups, an age-related increase in tonic and delayed firing neurons with a concurrent decrease in single spikers was observed. However, there were no significant differences in the proportions of the discharge categories between saline- and LPS-treated, or between the ipsi- and contralateral SDHs in any of the three age groups.

as the currents were obscured by AP discharge during the depolarizing step. In the remaining SDH neurons, one of four major subthreshold currents could be identified: either rapid A (I_{Ar}), slow A (I_{As}), the T-type calcium current (I_{Ca}), or the non-specific cationic current (I_H) (**Figure 3**, traces in upper panels). At PND 7 and 22, I_{Ar} was the dominant current on both the ipsi- and contralateral sides of the SDH, regardless of treatment group (saline or LPS). At PND 13, however, the proportion of subthreshold currents was more equitable between I_{Ar}, I_{As}, and I_{Ca}. The non-specific cationic current, I_H, was rarely observed in any of the groups in our study. A comparison of the proportions of subthreshold currents revealed no developmental differences in either saline- and LPS-treated rats (G *statistic* = 7.26, $p = 0.20$ and G *statistic* = 6.69, $p = 0.24$, respectively). Moreover, there was no difference in the proportions of subthreshold currents between the two treatment

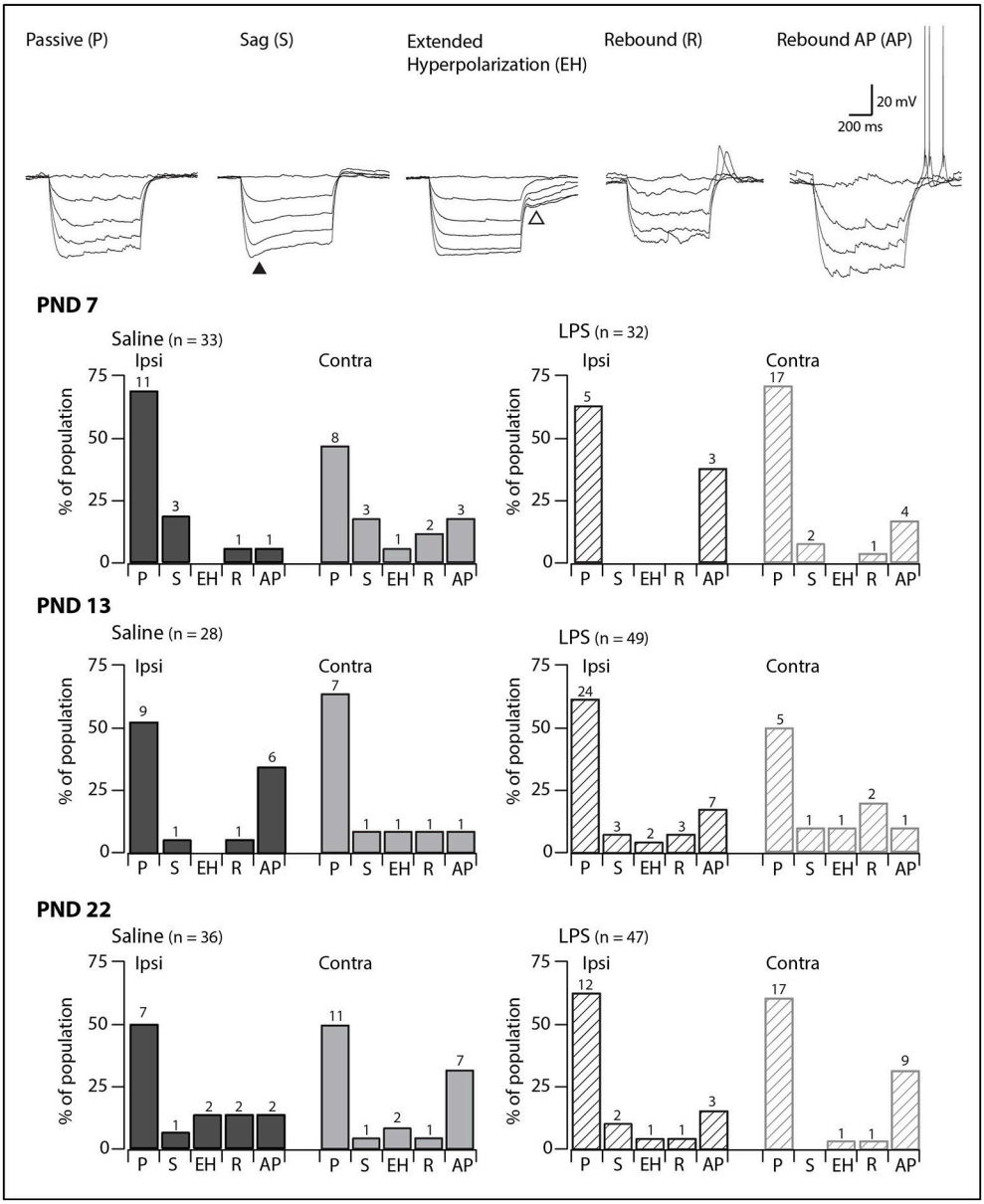

FIGURE 2 | Prevalence of responses to hyperpolarizing current injection. Upper traces: representative traces showing the five types of responses to hyperpolarizing current injection observed in SDH neurons. All traces are from saline-treated PND 22 SDH neurons (holding potential −60 mV). Closed triangle indicates the "sag" observed during hyperpolarizing current injection. Open triangle indicates the extended hyperpolarization observed upon release from hyperpolarization. Lower bar plots: demonstrate the prevalence of responses to hyperpolarizing current injection at the ages examined (PND 7, 13, and 22, *n* values for each bar indicated). SDH neurons were recorded both ipsilateral (dark gray) and contralateral (light gray) to formalin injection in animals pre-treated with either saline (solid bars) or LPS (hashed bars) as neonates. In both treatment groups, we observed an age-related decrease in passive responses, with a parallel increase in more complex responses. However, there were no significant differences in the proportions of the responses to hyperpolarizing current between saline- and LPS-treated, or between the ipsi- and contralateral SDHs at any of the three ages examined.

groups at any age examined (PND 7: *G statistic* = 4.40, $p = 0.49$; PND 13: *G statistic* = 4.05, $p = 0.54$; and PND 22: *G statistic* = 4.32, $p = 0.50$; **Figure 3**, bar plots). Therefore, neonatal exposure to LPS has no impact upon the presence and proportions of subthreshold currents in the SDH during development.

Properties of the Rapid A (I_{Ar}) Current

Since I_{Ar} was the dominant current across our sample, we compared its peak amplitude, time to peak and decay time constant across all groups in our study. The distribution of discharge categories from this subset of neurons mirrored the distribution of the overall population (as displayed in **Figure 1**),

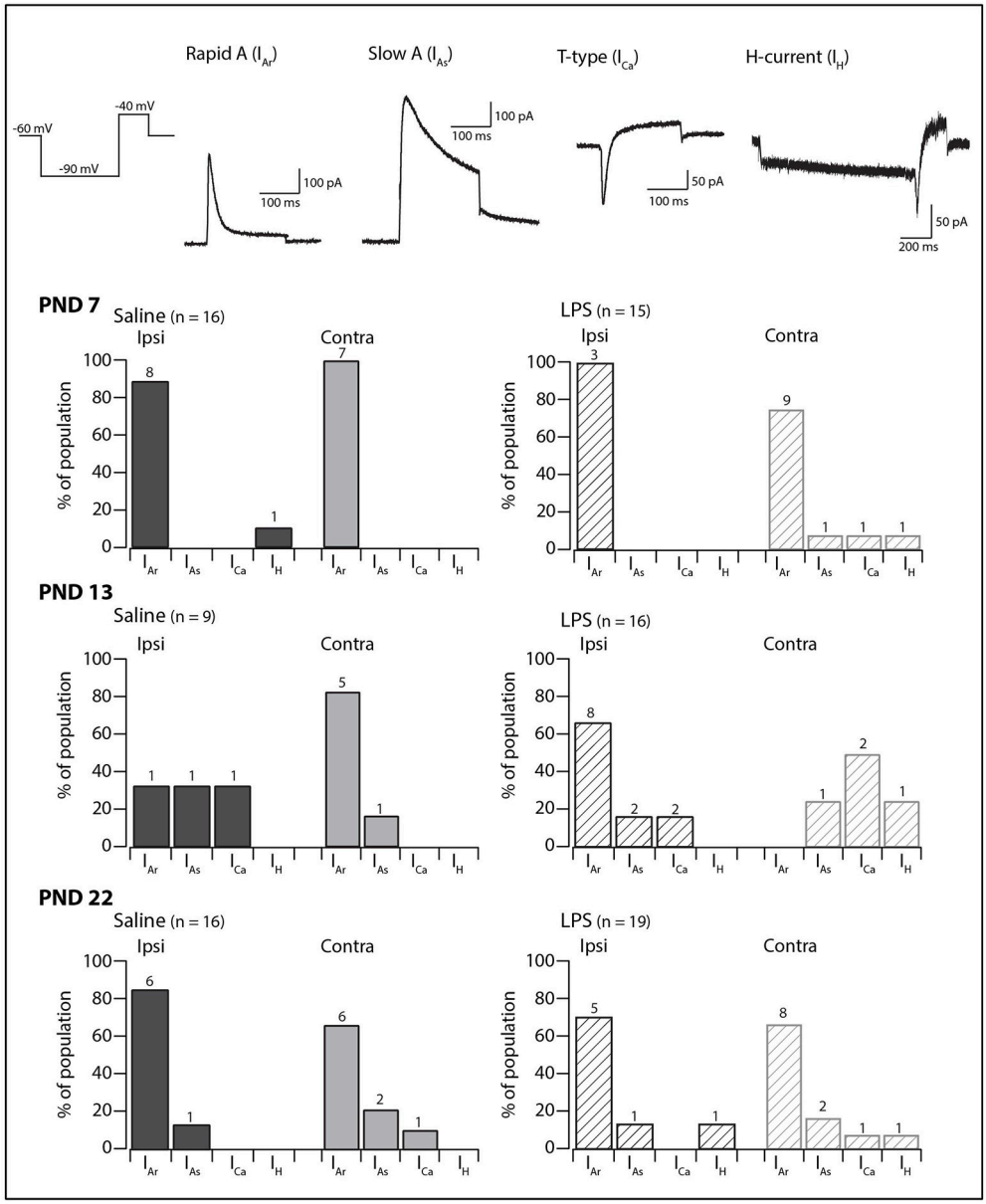

FIGURE 3 | Prevalence of subthreshold currents. Upper traces: representative traces showing the subthreshold currents observed in SDH neurons in response to a two-step voltage protocol (far left). All traces are from saline-treated PND 22 SDH neurons, recorded from a holding potential of −60 mV, with the exception of the H-current (I_H) which was recorded in an LPS-treated PND 22 SDH neuron. Lower bar plots: demonstrate the prevalence of AP discharge categories at the ages examined (PND 7, 13, and 22, *n* values for each bar indicated). SDH neurons were recorded both ipsilateral (dark gray) and contralateral (light gray) to the formalin injection site in animals pre-treated with either saline (solid bars) or LPS (hashed bars) as neonates. In both treatment groups, an age-related decrease in the rapid-A type current was observed. There were no significant differences in the proportions of the subthreshold current types between saline- and LPS-treated, or between the ipsi- and contralateral SDHs at any of the three ages examined.

therefore, represents an unbiased sampling. A two-way analysis of variance revealed a main effect for the peak amplitude of the I_{Ar} current [$F_{(1, 55)} = 4.517$, $p = 0.038$], with a significant interaction effect between the treatment and spinal cord location [$F_{(1, 55)} = 4.722$, $p = 0.034$]. Developmentally, we observed a significant increase in peak current between PND 7 and PND 22 (**Figure 4B**; saline ipsilateral —$p < 0.01$; LPS ipsilateral—$p = 0.02$), with no changes in either time to peak or decay time during this developmental period. Furthermore, we observed an increase in I_{Ar} peak amplitude in ipsilateral SDH neurons of the LPS- vs. saline-treated rats ($p = 0.04$). Peak I_{Ar} current amplitude also differed between ipsi- and contralateral SDH neurons in LPS-treated rats at both PND 7 ($p = 0.04$) and PND 22 ($p = 0.03$), with no corresponding changes in either time to peak or decay constant at either age. To account for any possible changes in channel distribution on the somato-dendritic

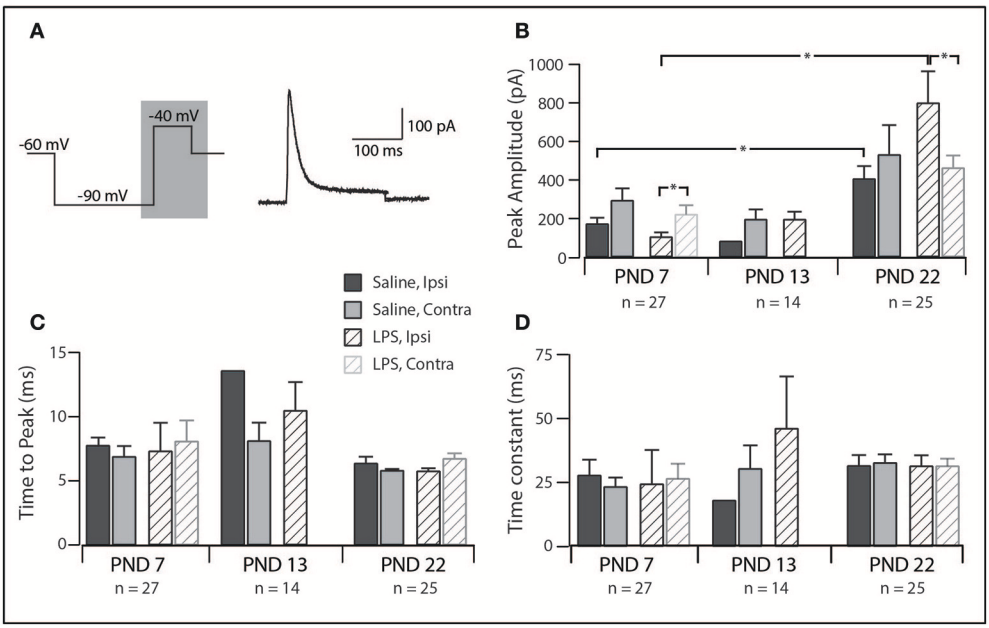

FIGURE 4 | Properties of the rapid-A subthreshold current. **(A)**, Left trace: two-step voltage protocol injected into SDH neurons to examine the presence of any subthreshold currents. Right trace: representative rapid-A current (from a saline-treated PND 22 SDH neuron) observed in response to the protocol on the left. Current trace taken from the area shaded in gray. **(B–D)**, Bar plots demonstrating selected rapid-A current properties for each treatment group, at each age group, both ipsi- and contralateral to the formalin injection. *indicates $p < 0.05$. **(B)**, Peak current amplitude showed an age-related increase and differed between ipsi- and contralateral SDH neurons at PND 7 and PND 22. **(C)**, time taken to reach peak current amplitude remained relatively stable, both between treatment groups and developmentally. **(D),** decay time constant did not change between ages or treatment groups.

tree of SDH neurons during development, we compared the membrane capacitance in all recorded neurons, and found no difference across any of the groups examined [$F_{(2, 218)} = 0.008$, $p = 0.992$]. Taken together, these data suggest the potassium channels responsible for generating the I_{Ar} current are maturing during the postnatal period between PND 7 and 22, and that this developmental trajectory appears to be susceptible to neonatal LPS exposure.

Spontaneous Excitatory Synaptic Currents

In order to assess network excitability in spinal cord slices, we recorded excitatory postsynaptic currents (sEPSCs; holding potential −70 mV) in a subset of SDH neurons (PND 7 = 50; PND 13 = 53; PND 22 = 57). The distribution of discharge categories from this subset of neurons mirrored the distribution of the overall population (as displayed in **Figure 1**), therefore, represents an unbiased sampling. sEPSCs represent the postsynaptic response to neurotransmitter release (both AP driven and quantal) from presynaptic terminals and are observed as downward deflections on the current traces (**Figure 5A**). We observed significant alteration of sEPSC parameters [frequency: $F_{(2, 148)} = 25.84$, $p = 0.00$; width: $F_{(2, 140)} = 4.78$, $p = 0.01$; decay: $F_{(2, 140)} = 4.49$, $p = 0.01$] with *post-hoc* comparisons revealing the changes described below. We observed a developmental increase in sEPSC frequency between PND 7 and 22, although this did not reach significance ($p = 0.06$). However, there was a significant increase in sEPSC width ($p < 0.01$) and decay time ($p < 0.01$) between PND 7 and 22. The combination of sEPSC properties produced an sEPSC charge transfer that was similar at all three ages [$F_{(2, 140)} = 0.563$, $p = 0.571$], however, the higher sEPSC frequency at PND 22 indicates an increased excitatory drive accompanies development. Taken together, these data demonstrate that the SDH is a highly plastic network during the first three postnatal weeks, with changes in both the number and location of synaptic connections within the SDH.

When studying the effect of neonatal exposure to LPS, we observed significant changes in the frequency of sEPSCs following formalin injection (**Figure 5B**). At PND 7, ipsilateral SDH neurons from LPS-treated rats displayed a lower sEPSC frequency ($p = 0.04$) and larger width and decay time ($p = 0.04$) compared to ipsilateral SDH neurons from saline treated rats ($p = 0.04$). Interestingly, in saline treated rats, we also observed an increased sEPSC frequency in ipsilateral compared to contralateral SDH neurons ($p = 0.01$). There were no differences in any sEPSCs parameter observed at PND 13. At PND 22, sEPSCs from LPS-treated rats exhibited a higher frequency ($p = 0.01$) and greater amplitude ($p = 0.02$) in ipsilateral vs. contralateral SDH neurons, however there was no significant differences between rats pre-treated with saline compared to LPS. Since there were no differences in the charge transfer at either PND 7 or 22, these changes in frequency indicate an increase in excitatory drive associated with neonatal LPS treatment. Taken with the developmental changes in the SDH described above, these data suggest neonatal exposure to

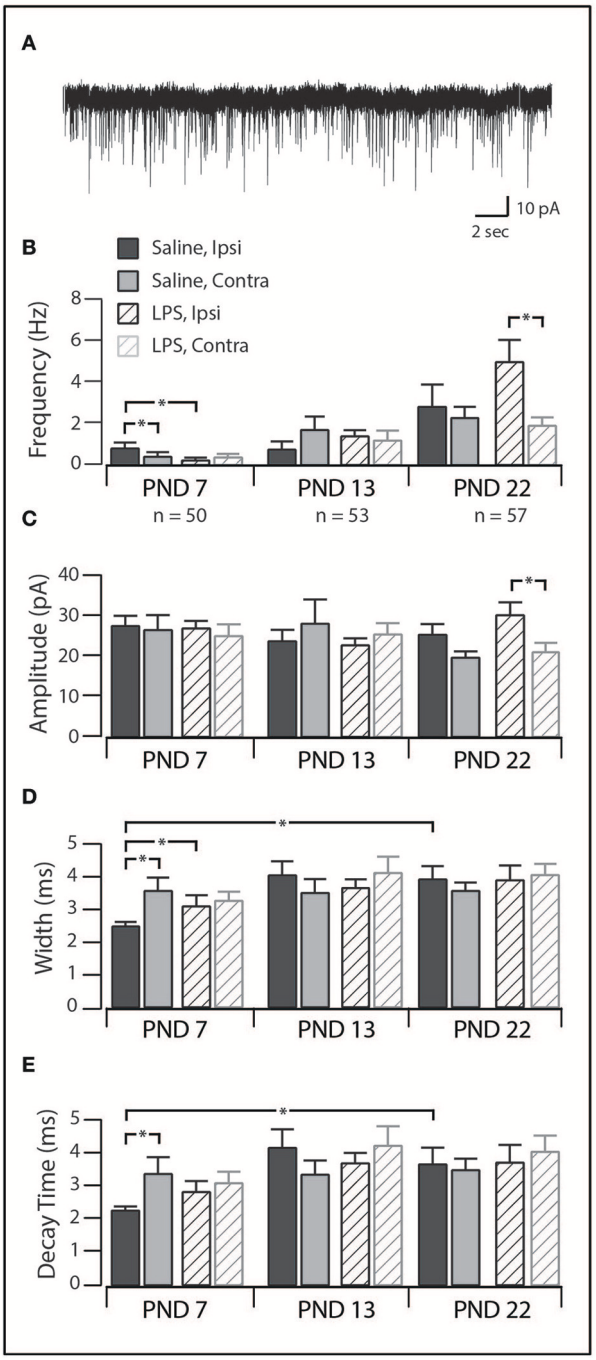

FIGURE 5 | Excitatory synaptic drive in SDH neurons. **(A)**, trace showing representative spontaneous excitatory postsynaptic currents (sEPSCs, 30 s of continuous data). Each downward deflection represents one sEPSC from a saline-treated PND 22 SDH neuron (holding potential −70 mV). **(B–E)**, Bar plots demonstrating selected sEPSC properties for each treatment group, at each age, both ipsi- and contralateral to the formalin injection. *indicates $p < 0.05$. **(B)**, sEPSC frequency displayed an age-related increase, as well as treatment-specific differences at PND 7 and PND 22. **(C)**, sEPSC amplitude remained relatively stable throughout development in both treatment groups. **(D)**, sEPSC width remained relatively stable throughout development in both treatment groups. **(E)**, sEPSC decay time remained relatively stable throughout development in both treatment groups.

LPS affects synaptic activity and thus SDH network excitability. Importantly, these changes are expressed upon exposure to a subsequent inflammatory event.

DISCUSSION

In this study, we examined the electrophysiological properties of SDH neurons in rats exposed to both a bacterial mimetic during the early neonatal period and a later inflammatory challenge at three time points during postnatal development. In addition to examining the long-term effects of neonatal inflammation, we were also able to map the developmental trajectory of SDH neurons in the rat by studying those treated with saline. Overall, we found no changes in the types or proportions of neuronal responses to either depolarizing or hyperpolarizing current. In contrast, we found the amplitude of the rapid A-type potassium increased both developmentally, and in response to neonatal treatment with LPS. We also observed significant changes in SDH network excitability, as measured by sEPSCs properties after a subsequent inflammatory event. Taken together, these data suggest a neonatal immune challenge does not markedly affect the intrinsic properties of SDH neurons, however, it can increase the excitability of local spinal cord networks via altering the properties of their rapid A currents and excitatory synaptic connections. These changes in neurons within spinal cord pain circuits have the capacity to alter nociceptive signaling in the ascending pain pathway.

Effect of Neonatal LPS Exposure on SDH Neuron Properties

We were surprised not to observe any long-term changes in SDH neuron responses to current injection following neonatal LPS exposure. Although our recordings were made several hours following formalin injection, prior studies have demonstrated that formalin injection into the hindpaw can have long-lasting effects. For example, behavioral studies show an increase in thermal and mechanical sensitivity 4 weeks after formalin injection (31) and extracellular recordings of dorsal horn neurons *in vivo* revealing an increase in AP discharge at least 80 min after formalin injection (32). Although studies on the developmental of rat SDH neurons are limited, we have previously identified a "critical period" during mouse SDH neuron development, between PND 6 and PND 10 (11). Furthermore, the HPA axis in rats is known to undergo a "stress hyporesponsive period" (SHRP) from PND 4 to 14 (33), where behavioral responses to the formalin test are blunted (3). Perhaps our LPS challenge, administered at PND 3 and 5, fell within a similar developmental "hyporesponsive period" in the rat SDH, and thus the effect on the responses to current injection was limited. Moreover, it is not known how long the central sensitization resulting from formalin injection persists *in vitro*, especially given the removal of peripheral and descending inputs during the slicing process.

Importantly, most studies on the effect of pro-inflammatory substance levels in the spinal cord have been undertaken in adult animals (8–10) and thus do not consider neuronal plasticity and the differing response of young and adult nervous systems to

antigens (34). For example, prostaglandin E2 (PGE2) levels are higher in neonatal versus adult plasma (35). This is interesting as PGE2 is known to modulate cytokines levels (36) and directly depolarize adult dorsal horn neurons when applied to spinal cord slices (6). In addition, whilst peripheral inflammation following formalin injection is known to last up to 4 weeks in the adult hindpaw (31), it is unknown how long this peripheral inflammation persists during the early postnatal period. Together these findings suggest further examination of the neuro-immune interface in the spinal cord at specific stages during development is required to better understand the mechanisms underlying long-term effects of neonatal immune challenges.

The amplitude of the rapid A potassium current increased during development. This suggests the number of channels responsible for generating the rapid A current at PND 22 following neonatal LPS exposure are increased (**Figure 4**). The rapid A current has been implicated in several developmental disorders (37) and is known to inhibit neuron activity by hyperpolarizing neurons and thus reducing the likelihood of AP discharge (25, 38). Short duration neonatal hypoxia (14–16 min) has been shown to result in a reduction of potassium channel mRNA levels as well as peak current amplitude in hippocampal cells (39). This indicates systemic insults can alter potassium channel expression within central neurons. In our study, the increased amplitude of the rapid-A-type potassium current following neonatal exposure to LPS could be a compensatory mechanism to balance out factors that would increase excitability, such as the increase in sEPSC frequency, within the SDH (38). Several studies have associated the rapid-A-type potassium current with the delayed firing AP discharge phenotype (38). Delayed firing, however, was rarely observed in our sample of neurons. This is best explained by reference to a recent computational study showing that the density and relative ratios of potassium channels can alter the phenotypical AP discharge pattern of SDH neurons (40, 41). Given our findings of altered channel expression during development, it is possible that the critical level of I_{Ar} channels required for delayed firing has not yet been reached and may continue to rise during the subsequent postnatal period.

At PND 22 we observed an increase in sEPSC frequency, width and decay time in SDH neurons from saline-treated rats, with an augmentation of this increase in frequency observed following neonatal exposure to LPS (**Figure 5**). An increase in sEPSC frequency is thought to indicate increased release probability or an increased number of synaptic connections. One possible factor that determines sEPSC width and decay time is the distance between the location of the synapse and the cell soma (42, 43). Given the increased primary afferent connections within the dorsal horn (44) and increased dendritic-arbor size in SDH neurons during development (45) it is perhaps not surprising that we noted increases in sEPSC width and decay time. Previous studies investigating the long-term impact of neonatal inflammation have demonstrated an augmentation of the developmental increase in the size of the terminal field of the sciatic nerve following injection of carrageenan into the neonatal hindpaw (12). This supports the hypothesis that the properties of the peripheral nervous system can be altered following a neonatal insult. Whilst our model of early-life event involved a systemic, rather than localized, insult, it is also probable that the DRGs of our LPS-challenged rats become "primed" during inflammation within the neonatal period. Indeed, studies in adult animals have shown DRG excitability can increase following a noxious insult (46, 47). Our data suggests exposure to another inflammatory attack (i.e., formalin) can transfer this increased peripheral signaling into the SDH and drive long-term changes in both SDH neuron properties and pain behavior. However, given the potential for corticosterone to alter GABAergic synaptic transmission within the dorsal horn (48), investigation of the inhibitory postsynaptic currents is required to determine the full impact of neonatal LPS exposure on synaptic transmission within the SDH.

Age-Related Changes in SDH Networks

It is well known that neuronal populations in various regions of the nervous system are highly plastic during early postnatal development (49–51). Surprisingly, limited data exist on the developmental trajectory of the spinal neurons (i.e., those in the SDH) that are crucial for pain signaling. To date, the most comparable study on the excitability of rat SDH neurons reported relatively stable excitability during the first three postnatal weeks (52). The findings in our study, however, show that SDH neuronal responses to current injection (**Figures 1**, **2**) and certain sEPSCs properties (**Figure 5**) are indeed altered during the first three postnatal weeks. These discrepancies could be attributed to differences in methodologies, with the prior study using perforated patch clamping, compared to our whole-cell recordings. Moreover, the previous study conducted recordings from a pre-determined membrane potential (−60 to −65 mV), whereas all recordings in our study were carried out from the neuron's RMP. Therefore, the reasons for the differences between this previous study and our own remain to be determined.

Importantly, the developmental findings outlined in this study demonstrate similarities with those from other species. For example, murine SDH neurons show changes in the proportion of various types of AP discharge during postnatal development. Most notably, the proportion of tonic firers increases, the proportion of single spikers decrease, and the responses of neurons to hyperpolarizing current becomes more complex during the early postnatal period (11). Moreover, these developmental changes in the responses to current injection are also observed in human SDH neurons (28). Thus, our data suggest the developmental trajectory of rat SDH neurons mimics that of other commonly used laboratory rodents, and, importantly, the development of human SDH neurons. The relevance of animal models to human conditions has been a topic of discussion in recent years (53, 54), with translational failures raising questions about interspecies differences and their relevance, especially in the ascending pain pathway. Given the findings that extreme preterm birth in humans (>26 weeks gestation) is associated with complex, centrally mediated alterations in sensory processing (55) the importance of appropriate laboratory models is critical. The similarities between the developmental trajectory of the rat SDH neurons in this study and those reported previously for both mouse (11) and

human (28) suggest our model of neonatal inflammation is appropriate for the investigation of the long-term impact of early life events.

Long-Term Implications of LPS Exposure in the Highly Heterogeneous SDH

Our previous studies (3, 4), and others (12, 56, 57), have clearly shown changes occur in the central nervous system in response to a neonatal immune challenge. However, in our study, the changes observed in SDH neurons following neonatal exposure to LPS were subtle. The data presented in **Figure 1** through 3 show the SDH is a highly heterogeneous neuronal population with at least five functional types based on AP outputs. It is now well appreciated that, in the adult SDH, these distinct AP discharge categories display a unique suite of ion channels and receptors that not only shape their AP discharge patterns, but also impact their roles within the pain neuroaxis [for review see: (58)]. Whilst in this study we were unable to examine the impact of neonatal LPS exposure on each discharge categories, it is possible that some categories may have been preferentially targeted and thus biased our sample. Indeed, previous work on the long-term effects of BDNF on SDH neurons clearly demonstrated changes within these subpopulations, including a decrease in excitatory drive to tonic firers, but an increase for all other discharge categories, indicating a neuron-specific response (59). Thus, to further elucidate the role of SDH neurons in the altered pain behaviors observed in our model, future studies should target specific subpopulations (60). This would help determine whether any subpopulation is preferentially altered by neonatal exposure to LPS and thus represent a future drug target.

CONCLUSIONS

It is now well accepted that extensive cross-talk exists between the immune and nervous systems, and that early life events can have long-term programming effects on the neuroimmune status (61, 62). We have previously reported developmentally regulated long-term changes in the neural, endocrine, and behavioral responses to inflammatory pain following neonatal LPS exposure (3, 4). The present study builds on these prior findings, by demonstrating subtle, but potentially important, differences in the susceptibility of SDH neurons to noxious inflammatory stimuli later in life. This provides a possible mechanism through which neonatal LPS exposure enhances pain responses later in life.

AUTHOR CONTRIBUTIONS

MT, IZ, DH, and RC conceived and designed the experiments. MT and IZ conducted the experiments, analyzed the data, and prepared the manuscript. All authors approved the final version of the manuscript.

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REVIEW
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From the Bottom-Up: Chemotherapy and Gut-Brain Axis Dysregulation

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The central nervous system and gastrointestinal tract form the primary targets of chemotherapy-induced toxicities. Symptoms associated with damage to these regions have been clinically termed chemotherapy-induced cognitive impairment and mucositis. Whilst extensive literature outlines the complex etiology of each pathology, to date neither chemotherapy-induced side-effect has considered the potential impact of one on the pathogenesis of the other disorder. This is surprising considering the close bidirectional relationship shared between each organ; the gut-brain axis. There are complex multiple pathways linking the gut to the brain and vice versa in both normal physiological function and disease. For instance, psychological and social factors influence motility and digestive function, symptom perception, and behaviors associated with illness and pathological outcomes. On the other hand, visceral pain affects central nociception pathways, mood and behavior. Recent interest highlights the influence of functional gut disorders, such as inflammatory bowel diseases and irritable bowel syndrome in the development of central comorbidities. Gut-brain axis dysfunction and microbiota dysbiosis have served as key portals in understanding the potential mechanisms associated with these functional gut disorders and their effects on cognition. In this review we will present the role gut-brain axis dysregulation plays in the chemotherapy setting, highlighting peripheral-to-central immune signaling mechanisms and their contribution to neuroimmunological changes associated with chemotherapy exposure. Here, we hypothesize that dysregulation of the gut-brain axis plays a major role in the intestinal, psychological and neurological complications following chemotherapy. We pay particular attention to evidence surrounding microbiota dysbiosis, the role of intestinal permeability, damage to nerves of the enteric and peripheral nervous systems and vagal and humoral mediated changes.

Keywords: chemotherapy-induced cognitive impairment, mucositis, chemotherapy-induced gut toxicity, gut-brain axis, microbiota

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INTRODUCTION

The chemotherapy experience is associated with powerful psychological, neurological and somatic side-effects. Cancer diagnosis and the complications arising from treatment induce anxiety and depression, fatigue, pain, and cognitive impairments while patients struggle to maintain hope for recovery and continue normal daily functions, routines and roles (Kuzeyli Yildirim et al., 2005; Downie et al., 2006; Chan et al., 2014). Due to the non-selective and systemic nature of most chemotherapy drugs,

they also target healthy, rapidly-dividing non-malignant cells. The regions of the body most susceptible to the unwanted toxicities of chemotherapy exposure are the gastrointestinal tract (GIT) and the central nervous system (CNS)—the gut and brain. Many chemotherapy drugs are small enough to readily cross the blood-brain barrier and result in molecular, structural and functional changes within the CNS, manifesting as cognitive changes in a subset of patients (Wigmore, 2012). Outside of the CNS, the cells of the GIT are particularly vulnerable to damage following chemotherapy exposure. In particular, epithelial cells within the mucosal layer lining the alimentary tract form prime targets due to chemotherapy drugs targeting proliferating enterocytes (Sonis, 2004). Although the gut and the brain appear disparate, they are intimately connected. The complex network of pathways linking the gut to the brain will be discussed in more detail below as we present mechanisms by which chemotherapy results in gut-brain axis dysregulation.

This network has a bidirectional relationship. For instance, psychological and social factors have the ability to influence motility and digestive function, symptom perception, behaviors associated with illness and the pathological outcome (Bhatia and Tandon, 2005). On the other hand, visceral pain affects central pain perception and pathways, mood and behavior (Chakiath et al., 2015). Importantly, systemic and gut immunity is tightly regulated by the inflammatory reflex and cholinergic anti-inflammatory pathway (Tracey, 2002; Pavlov and Tracey, 2012). Integral components of the inflammatory reflex include innate immune cell activation, release of inflammatory mediators, such as cytokines, vagal innervation and responses from higher order brain regions, such as the nucleus tractus solitarius. Vagal innervation is of particular importance in the chemotherapy setting as it is pivotal in the transmission of chemo and mechanosensory information from the gut to the brain (**Figure 1**; Goehler et al., 2000; Tracey, 2002). In this sense, proinflammatory mediators and cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) activate primary afferent nerve fibers within the vagal sensory ganglia. Vagal ganglia signal several nuclei of the dorsal vagal complex responsible for the integration of visceral sensory input and relay information to higher order brain regions like the hypothalamus, hippocampus and forebrain. Coordinated autonomic and behavioral responses are initiated to assist in restoration of homeostasis. Importantly, efferent vagal motor activity inhibits cytokine synthesis, creating the inflammatory reflex effect. Humoral anti-inflammatory pathways can be activated, stimulating the release of adrenocorticotrophin hormone. Sympathetic outflow can also increase localized adrenaline and noradrenaline expression and further suppress inflammation. The activation of these innate components of the inflammatory reflex, including the vagally-mediated cholinergic efferent output, ultimately results in the regulation of systemic and localized inflammation, having important implications in gut immunity (**Figure 1**). A more comprehensive outline of the inflammatory reflex has been reviewed elsewhere (Tracey, 2002; Pavlov and Tracey, 2012).

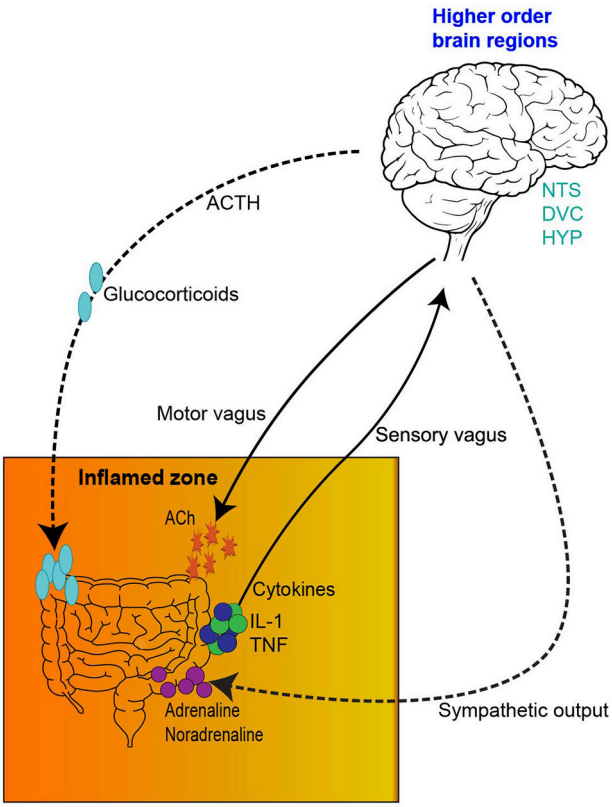

FIGURE 1 | The inflammatory reflex. (1) The inflamed zone represents tissue damage, infection and ischemia. (2) Increased expression of inflammatory mediators and cytokines, such as Il-1β and TNF-α are released by cells in the inflamed zone. (3) Cytokines activate primary afferent neurons within the vagal sensory ganglia. (4) Afferent visceral signals are relayed to nuclei in the dorsal vagal complex (DVC), such as the nucleus tractus solitarius. (5) Visceral information is further relayed from the DVC to higher order brain regions, such as the hypothalamus, hippocampus and forebrain. (6) Activation of efferent vagal motor activity inhibits cytokine synthesis. (7) Hypothalamus activation stimulates the release of adrenocorticotrophic hormone from the pituitary gland, initiating a humoral anti-inflammatory pathway. (8) Sympathetic outflow can increase localized adrenaline and noradrenaline expression further suppressing inflammation. IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor—alpha; DVC, dorsal vagal complex; NTS, nucleus tractus solitarius; HYP, hypothalamus; HIP, hippocampus; FB, forebrain; ACTH, adrenocorticotrophic hormone.

Additionally, activation of the neuroimmune system via glial priming and neurogenic inflammation further complicates immune to brain signaling. Although glial cells are non-neuronal cell types which can be found in the CNS and periphery, such as oligodendrocytes and Schwann cells, for the remainder of this manuscript we specifically refer to microglia and astrocytes. For an in depth analysis of glial priming and neuroinflammation several excellent reviews exist (Araque et al.,

Abbreviations: CICI, Chemotherapy-induced cognitive impairment; ENS, enteric nervous system; PNS, peripheral nervous system; EGC, enteric glial cells; IBS, irritable bowel syndrome; IBD, inflammatory bowel diseases; FGIDs, functional gastrointestinal disorders; BBB, blood-brain barrier; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumour necrosis factor alpha; 5-FU, 5-Fluorouracil; DSS, dextran sodium sulfphate; CIGT, chemotherapy-induced gut toxicity; TLRs, toll-like receptors; CIPN, chemotherapy-induced peripheral neuropathy; 5-HT, serotonin 5-hydroxytryptamine; DCs, dendritic cells.

1999; Bains and Oliet, 2007; He and Sun, 2007; Allen and Barres, 2009; Capuron and Miller, 2011; Parpura et al., 2012; Dodds et al., 2016). Nonetheless, to illustrate this point in the context of cancer and chemotherapy, inflammation (either centrally or locally derived from either the malignancy or chemotherapy) and the release of proinflammatory cytokines signals the brain and activates neuroimmunological cells, glia (**Figure 2**). Proinflammatory cytokines access the brain either directly via leaky circumventricular organs or indirectly via a neural route (e.g., vagal transmission). Microglia and astrocytes form an integral part of the tri- and tetra-partite synapses and form a close bidirectional relationship with neurons; the neuroimmune interface which has wide implications in central health and disease (Allen and Barres, 2009; Graeber and Streit, 2010; Grace et al., 2014; Dodds et al., 2016). Reactive glia undergo morphological changes and overproduce proinflammatory mediators whilst reducing anti-inflammatory output (O'Callaghan et al., 2008; Agrawal and Yong, 2011). Ultimately, glial reactivity results in a neuroinflammatory environment whereby neurotoxicity causes damage to surrounding tissues and neurons (Eikelenboom et al., 2006; Bilbo et al., 2012; Laskaris et al., 2015). Centrally derived neurogenic inflammation and signaling also contributes to the exacerbation of peripheral inflammatory conditions. Although glial reactivity may begin with beneficial intentions by responding to insults (disease, trauma, infection or drug exposure), glia may remain in a primed state and be sensitized even after the initial insult has resolved, eliciting an exaggerated immune responses (**Figure 2**). Critically, in particular brain regions primed glia and neuroinflammation influence behaviors involving cognition and are involved in the pathogenesis of various neurodegenerative diseases and pathological pain states (McGeer et al., 1988; Eikelenboom et al., 2006). Due to the altered immune profile of cancer and chemotherapy patients, it has been suggested that neuroinflammatory processes may be contributing to the cognitive deficits often experienced by this patient group (Myers, 2009; Johnston, 2014). This form of innate immune (peripheral-to-central) signaling represents a plausible mechanism meditating chemotherapy-induced gut toxicity and neurological changes (**Figure 2**).

Following on from this, it is not surprising that interactions between the immune system and the brain become dysregulated under cancer and chemotherapy conditions. Further, recent evidence has highlighted the impact gut commensal bacteria has in both central and peripheral development and health (Feng et al., 2018). Importantly, dysbiosis (microbial imbalance/maladaptation) and gut-brain axis dysfunction have been associated with functional gut disorders having negative effects on cognition (Jones et al., 2006; Frank et al., 2007). Previously, research has focussed on a single pathological manifestation of chemotherapy exposure, for example gut toxicity or regional structural brain changes (Keefe et al., 1997; Christie et al., 2012). Such studies have failed to consider the *indirect* effects of simultaneously occurring treatment-induced toxicities, which may be contributing to the primary pathology under investigation. Consequently, we hypothesize that chemotherapy treatment causes severe and prolonged psychosocial impacts on the survivor. Furthermore, we suggest that the gut-brain axis is an important mediator of a diverse range of cognitive and emotional disorders similar to those experienced by cancer survivors. Here, we will determine whether chemotherapy affects the gut-brain axis and present several key stages. Following on from this, we suggest that the psycho-social side effects of chemotherapy treatment could be caused by the effects of chemotherapy on the gut-brain axis.

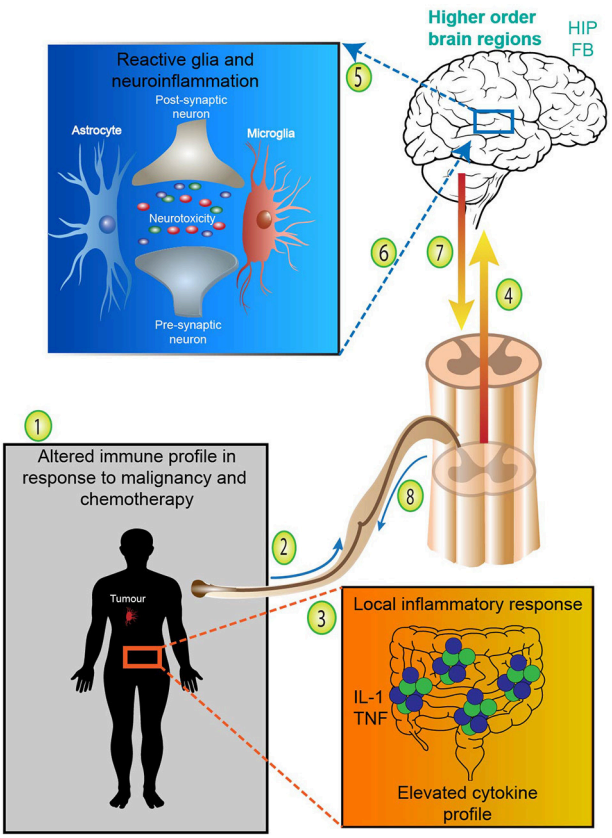

FIGURE 2 | Adapted from Dodds et al. (2016). Neuroimmunological complications arising from cancer and chemotherapy treatment. (1) Cancer patients undergoing chemotherapy treatment express an altered immune profile with increases in proinflammatory cytokines. (2) Systemic proinflammatory cytokines and mediators, such as IL-1 and TNF released either from the malignancy or treatment-associated toxicities access the brain directly via leaky circumventricular organs or (3) indirectly via neural transmission. (4) Systemic or localized proinflammatory mediators and cytokines signal higher order brain regions and (5) activate microglia and astrocytes. Reactive glia undergo morphological changes and overproduce proinflammatory mediators whilst reducing anti-inflammatory output—resulting in neurotoxicity and neuroinflammation. (6) In particular brain regions primed glia and neuroinflammation influence behaviors involving cognition and contribute to various neurodegenerative diseases. (7) Centrally derived neurogenic inflammation and descending signaling in the spinal cord (8) contributes to the exacerbation of peripheral inflammatory conditions and exaggerated pain states. Therefore, peripheral-to-central innate immune signaling represents a plausible mechanism meditating chemotherapy-induced gut toxicity and neurological changes. FB, forebrain; HIP, hippocampus; IL-1, interleukin-1; TNF, tumor necrosis factor.

Following a brief analysis of gut-brain communication, we will review some key studies linking gut-brain axis dysregulation to specific psychiatric disorders, highlighting similarities between these conditions and the chemotherapy setting. From the bottom-up (GIT to the brain) we will examine chemotherapy-induced gut and central changes and present several mechanisms meditating gut-brain axis dysregulation in the chemotherapy setting; focussing on the microbiome, intestinal integrity, peripheral neuron and enteric nervous system (ENS) dysfunction. Finally we will address the role vagal-, neural-, and humoral-mediated responses may play in these complex chemotherapy-induced pathological conditions. Overall, we aim to illustrate the complex role gut-brain axis dysregulation plays in shaping neurological changes associated with chemotherapy exposure.

GUT BRAIN CROSSTALK

Since Pavlov's Nobel Prize-winning discovery on the role neural innervation plays in gastric secretion—the first functional evidence connecting the gut and brain—our understanding of the pathways connecting the CNS and the GIT have significantly advanced (Keller and William, 1950). The multiple bidirectional pathways responsible for controlling signaling from the brain to the gut and vice versa have been extensively reviewed and is outside the scope of this manuscript (Mayer, 2011; Al Omran and Aziz, 2014; Carabotti et al., 2015; Furness, 2016). The complexity of this network is best appreciated in its ability to integrate information from a variety of systems encompassing the central, autonomic and enteric nervous systems (including the influence of the intestinal microbiota), whilst simultaneously considering neuroendocrine, enteroendocrine and neuroimmune input (summarized in **Figure 3**; Carabotti et al., 2015). A brief analysis of bottom-up and ENS mechanisms is necessary to appreciate the systems by which the integration of these pathways influence behavior and impact central comorbidities in disorders of the gut. We begin this section from the bottom-up; presenting key pathways, cell types and signaling mechanisms involved in communication from the gut to the brain. We also illustrate mechanistic evidence relating to disorders of the gut which often have a central comorbidity component, such as in the case of inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). Whilst research covering the central comorbidities associated with IBD and IBS continues to expand, the potential mechanisms linking neurological and gut changes following chemotherapy exposure remains under investigated.

From the Bottom-Up

The GIT elicits a myriad of functions ultimately resulting in absorption of nutrients and expulsion of noxious chemicals and pathogens via muscular contractions, cellular, endocrine and immune mechanisms. Critically, the gut harbors a diverse microbial community (bacteria, fungi, archaea, viruses, and protozoa) and has prolific central effects mediating a healthy host (Feng et al., 2018). Consequently, changes in gut-microbial composition disrupts physiological homeostasis, often contributing to central maladaptations (Mu et al., 2016; Dinan and Cryan, 2017). Recent advances in our understanding of the impact the microbiota has on the gut-brain axis has led to common use of the term *microbiota-gut-brain axis* (Rhee et al., 2009; De Palma et al., 2014). Microbiota-gut-brain axis communication alters certain aspects of brain development, function, mood and cognitive processes from both the bottom-up and top-down (Catanzaro et al., 2014; De Palma et al., 2014; Mayer et al., 2014; Tillisch, 2014; Carabotti et al., 2015; Barbara et al., 2016). Evidence specifically related to chemotherapy-induced microbiota changes will be discussed further below (see reviews on microbiota-gut-brain axis; Rhee et al., 2009; De Palma et al., 2014; Mayer et al., 2014; Tillisch, 2014).

The GIT maintains an extensive intrinsic nervous system, the ENS which is unique in its ability to control certain functions

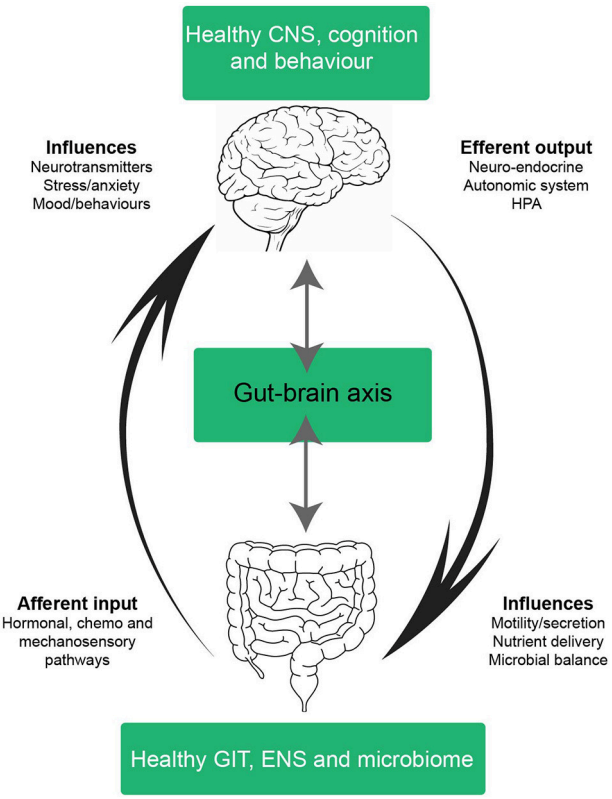

FIGURE 3 | Schematic of a healthy gut-brain axis. The arrows highlight the bidirectional nature of the gut-brain axis; in a balanced system mechanisms from the bottom-up (and vice versa) exist in cohesion. In a healthy system, the gut-brain axis integrates information from many systems; the central nervous system (CNS), autonomic nervous system (ANS), enteric nervous system (ENS), neuroendocrine, enteroendochrine, neuroimmune, and hypothalamic-pituitary axis (HPA). The complex bidirectional communication pathways and systems shared between the gut and the brain maintain health and homeostasis in the CNS, GIT, and microbiota. Efferent signals from the brain involving neuro-endocrine, autonomic and HPA outputs influence motility, secretion, nutrient delivery and microbial balance in the GIT. Whilst afferent inputs from the GIT, such as intestinal hormones, cytokines and sensory perceptions influence neurotransmitter expression, stress, anxiety, mood and behavior. CNS, central nervous system; ANS, autonomic nervous system; ENS, enteric nervous system; HPA, hypothalamic-pituitary axis; GIT, gastrointestinal tract.

of the small and large intestines even when it is disconnected from the CNS (Furness, 2016). However, the ENS should not be considered fully autonomous due to the constant top-down input it receives. The ENS is the largest and most complex division of the peripheral nervous system (PNS) comprising 400–600 million neurons and an extensive network of enteric glial cells (EGC) (Furness, 2012). EGCs share similarities with astrocytes, their CNS counterparts in the mechanisms they adopt to support enteric neurons, including their morphology, function and molecular capabilities (Gulbransen and Sharkey, 2009). Importantly, EGCs play key roles in mounting an immune response, particularly during intestinal inflammation.

Luminal environmental factors, such as mechanical and chemical changes are signaled from the gut to the brain via endocrine, immune and neuronal afferent pathways (Mayer, 2011; Furness, 2012; Al Omran and Aziz, 2014; Furness et al., 2014). Information regarding the level of distension, concentrations of specific nutrients, electrolytes, pH, and the presence of danger and immune signals is transmitted from the gut to the brain via a wide variety of neural and systemic communication pathways. Visceral changes are detected by a variety of sensory cell types including enterocytes, intrinsic and extrinsic primary afferent neurons, immune and enteroendocrine cells (Carabotti et al., 2015).

Hence, a wide variety of hormones and metabolites from the gut communicate homeostatic information to the brain via functional effector cells (enterocytes, smooth muscle cells, interstitial cells of Cajal, enterochromaffin cells, intrinsic and extrinsic primary afferent neurons, immune and enteroendocrine cells) (Carabotti et al., 2015). Examples of homeostatic information relayed from the functional effector cells include but are not exclusive to sensory, pH, water metabolism, chemical, danger and immune signals, etc.). Each cell type responds to luminal environmental changes and secretes specific signaling molecules which may include but are not exclusive to ghrelin, cholecystokinin, glucagon-like peptide-1, corticotrophin releasing hormone, proteases and cytokines, etc. (Furness et al., 2014). To further complicate gut-brain crosstalk, various neurotransmitters commonly produced centrally are also expressed in the GIT (Furness et al., 2014). Gut derived neurotransmitters, such as dopamine, serotonin and neuropeptide Y influence many aspects of central homeostasis, yet in the gut are responsible for appetite, satiety, hunger, pain and are implicated in the activation of reward pathways relating to food and beverage intake (Furness, 2016).

Numerous afferent and efferent pathways connect the gut and brain, presenting the host with a multitude of platforms for malfunction, dysregulation and disease, both in the periphery and centrally. Whilst the basic principles outlining top-down signaling have been extensively reviewed (Al Omran and Aziz, 2014; Furness et al., 2014; Furness, 2016) and is outside the scope of this review, it is crucial to acknowledge that these effects occur simultaneously with those described from the bottom-up. Importantly, top-down sympathetic and parasympathetic interactions suppress secretion, motility and GI transit, having direct effects on immune-, emotion-, mucosa-, and microflora-related alterations (Lyte et al., 2011; Mayer, 2011). Gut-brain axis dysfunction has played a pivotal role in our mechanistic understanding of various gut disorders and their effects on cognition. Indeed, experimentally induced gut disorders have critically developed our understanding of the mechanisms underlying central changes induced by disruptions in gut homeostasis. Disorders of the gut and chronic inflammation often result in psychological abnormalities, such as anxiety and depression (Nyuyki and Pittman, 2015). Additionally, physiological responses can be induced by stress, for instance triggering relapse in experimental colitis (Bernstein et al., 2010).

Great interest has recently been paid to the importance of gut health on mental health and vice versa. This has become particularly evident in the continual expansion of anecdotal evidence on the central comorbidities associated with various gut disorders, particularly in IBS and IBD (Drossman et al., 1999; Whitehead et al., 2002). Disorders of the gut are commonly associated with poorer mental health. For instance, 54–94% of IBS patients actively seeking treatment also present with emotional, psychological and cognitive comorbidities (Whitehead et al., 2002) as do chemotherapy recipients. The literature presented above provides clear evidence that gut disorders often occur simultaneously with central comorbidities, aligning with our hypothesis that gut-brain axis dysregulation may be mediating both chemotherapy-induced mucositis and neurological changes. Therefore, it is pivotal that we determine the *direct* and *indirect* central consequences of drug-induced gut disorders, such as chemotherapy-induced mucositis. Chemotherapy induces a range of peripheral and central side-effects, significantly reducing quality of life. In the gut this has been termed chemotherapy-induced mucositis and in the CNS, chemotherapy-induced cognitive impairment (CICI). The current review will now explore whether mucositis and CICI are linked and whether they exacerbate other symptoms, such as pain associated with mucositis, or cognitive impairment which are often experienced simultaneously in the chemotherapy setting.

CHEMOTHERAPY FROM THE BOTTOM-UP: THE GUT AND COGNITION

Chemotherapy drugs can be considered paradoxical at the most basic level. Primarily, they offer recipients' survivorship as they target malignant cells in an attempt to rid the host of cancer. On the other hand, due to their non-selective nature, they also target healthy cells and induce a range of side-effects reducing patient quality of life. The organ where their actions are perhaps often first noticed is the GIT due to its high regenerative capacity. Mucositis occurs in up to 70% of chemotherapy recipients and may manifest anywhere along the alimentary tract, termed oral or intestinal mucositis (**Figure 4**; Scully et al., 2003). It is one of the most significant dose-limiting side-effects of intensive anti-cancer therapy due to the painful nature of the disorder.

Sonis classified the pathogenesis of mucositis into five stages (Sonis, 2004). Hallmark characteristics of mucositis include villus atrophy, shallow crypts, inflammation and ulceration. Mucositis results in a high inflammatory response via the up-regulation

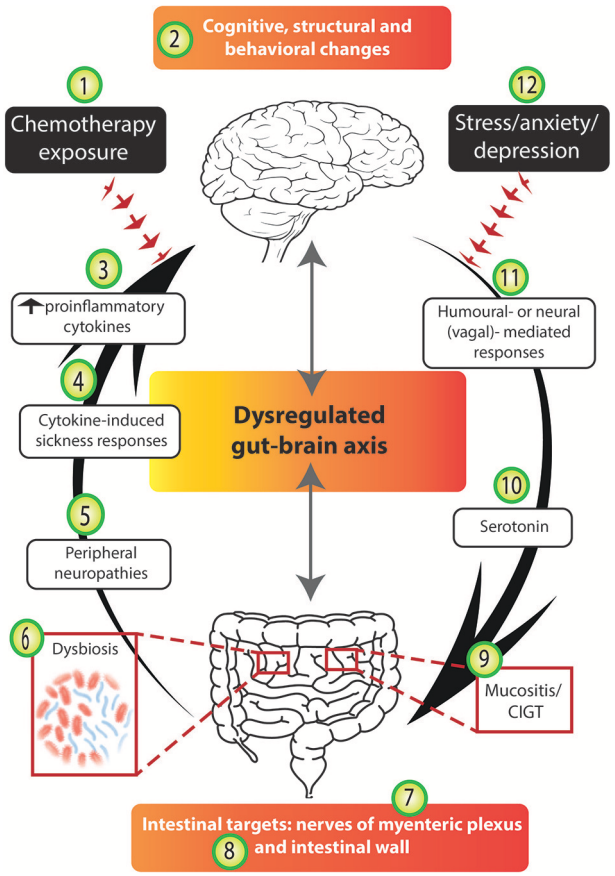

FIGURE 4 | Chemotherapy disrupts several stages of the gut-brain axis. The arrows highlight the bidirectional nature of the gut-brain axis; in an unbalanced system mechanisms from the bottom-up (and vice versa) are disrupted. We suggest that chemotherapy-induced gut-brain axis dysregulation plays a major role in the intestinal, psychological and neurological complications experienced by many cancer patients. Chemotherapy exposure (1) often results in molecular and structural changes in the brain (2), e.g., hippocampal changes as identified in rodent models. Chemotherapy exposure causes cognitive and behavioral changes (2) to a subset of patients and these findings have been supported by some experimental models. The altered immune profile of chemotherapy recipients results in increased circulating pro-inflammatory cytokines (3) which have been reported to cause cytokine-induced sickness-like responses (4) which mimic chemotherapy-induced side-effects. Damage to peripheral nerves resulting in peripheral neuropathies (5) are experienced by some chemotherapy recipients. Chemotherapy targets the intestines and its microbial contents causing dysbiosis (6), impairing the nerves of the myenteric plexus (7), damaging intestinal wall parameters (8), and resulting in mucositis (9). Serotonin dysregulation under chemotherapy conditions (10) may play a role in chemotherapy-induced intestinal and neurological changes. Finally, both humoral and neural/vagal peripheral-to-central immune signaling pathways (11) may mediate chemotherapy-induced gut-brain axis dysregulation. Importantly, we acknowledge that the stress, anxiety and depression associated with cancer diagnosis and treatment (12) may contribute to both bottom-up and top-down pathways, having negative effects in the gut and the brain.

and activation of various transcription factors, ultimately causing elevations in circulating proinflammatory cytokines (**Figure 4**), in particular IL-1β and TNF-α (Sonis, 2004). Whilst mucositis is an acute phenomenon which usually resolves upon cessation of chemotherapy treatment, clinical symptoms generally begin 5–10 days post-chemotherapy exposure and include significant pain, abdominal bloating, nausea and vomiting, diarrhea and/or constipation (Gibson and Keefe, 2006). Although guidelines for the prevention and treatment of mucositis exist, they fail to include effective treatment options (Gibson et al., 2013). Novel complementary treatment approaches are showing positive results utilizing naturally sourced products, such as Emu Oil and Rhubarb extract (Mashtoub et al., 2013; Bajic et al., 2016a). Although these treatment strategies show promise, to date they are still in the pre-clinical stages.

Our understanding of the central consequences of drug-induced gut disorders, such as mucositis remains elusive, yet evidence on CICI is expanding as various mechanisms underlying its pathogenesis are becoming clearer. CICI occurs in 15–45% of patients undergoing anti-cancer therapy (**Figure 4**; Vardy and Tannock, 2007). Subjective (self-report) report rates are considerably higher than objective measures with some studies reporting 95% of patients experiencing changes in cognitive performance (Downie et al., 2006). Subjective measures are nonetheless important as they identify the impact of cognitive impairment and the strain it places on patients' lives and daily functioning (Shilling and Jenkins, 2007). The breast cancer population forms the majority of the CICI literature as they offer researchers completion of extensive retrospective studies due to their typically good prognosis (Ahles et al., 2010). Regardless, CICI has been investigated in a range of other cancer types including myeloma and testicular cancer (Schagen et al., 2008; Potrata et al., 2010).

The cognitive domains most commonly reported in CICI are executive functioning, attention and concentration, processing speed, reaction time and motor speed and dexterity (Asher, 2011). Perceived cognitive impairment affects various facets of the patient's life, including relationships, employment, self-esteem/worth, finances and independence. The CICI experience leaves patients feeling distressed, anxious, frustrated, irritable, depressed and embarrassed, often reducing confidence (Mitchell, 2007; Von Ah et al., 2013). Current estimates on the duration of CICI are varied with some studies identifying deficits up to 20 years post-chemotherapy cessation, yet most indicate improvements up to 12 months later (Collins et al., 2009; Koppelmans et al., 2012). Neuroimaging studies have confirmed molecular and structural changes in the gray matter of the frontal and temporal lobes and the cerebellum of breast cancer patients following chemotherapy exposure (Silverman et al., 2007; McDonald et al., 2010). Additionally, chemotherapy induces white matter tract changes and the reorganization of global brain networks, which have undoubtable associative if not causal impacts on cognitive performance (Abraham et al., 2008; Bruno et al., 2012).

Animal studies have begun to unravel various mechanisms underlying the pathogenesis of CICI and involve structural and behavioral changes. Hippocampal and frontal cortical alterations have correlated with behavioral memory changes in various rodent models (**Figure 4**; Yang et al., 2010, 2012; Wigmore, 2012). Neurogenesis occurs in the dentate gyrus and cellular

proliferation is critical in hippocampal circuit plasticity and memory consolidation (Deng et al., 2010; Ming and Song, 2011). CICI models have reported on the vulnerability of stem cells to proliferate in the dentate gyrus irrespective of chemotherapy drug class (Briones and Woods, 2011; El Beltagy et al., 2012). Considering the pivotal role neural stem cells in this region have to divide into new neurons or astrocytes, disruptions in this process present as a direct mechanism which may be contributing to CICI. More recently, neuroimmunological manifestations, such as glial dysregulation and neuroinflammation, have been reported to contribute to CICI (Briones and Woods, 2013; Bajic et al., 2016b).

Currently, effective prevention strategies or treatment approaches for CICI remain undetermined although two evidence-based guidelines are available to assist oncologists in addressing cognitive deficits (Network, 2015). Other interventions for CICI are broadly categorized into cognitive training, compensatory strategies, pharmacological, and complementary and integrative medicines (Vance et al., 2017). Recently, Toll-like receptors (TLRs) have been suggested as a common component in the pathology of neuropathy/pain and chemotherapy-induced gut toxicity, presenting a novel and much needed therapeutic approach in the treatment of chemotherapy-induced toxicities (Wardill et al., 2015). TLRs have profound homeostatic effects, tightly regulating innate immune and gut functions, modulating pain behaviors (Akira and Takeda, 2004; Rakoff-Nahoum et al., 2004; Doyle and O'Neill, 2006; Hutchinson et al., 2010, 2012; Gibson et al., 2016). Wardill et al. (2015) hypothesized that TLR-4 mediates glial activation and neuropathy driven by the molecular signals released from chemotherapy-induced gut toxicity. Primary studies have indicated that an altered TLR expression profile may contribute to chemotherapy-induced pain and diarrhea (Gibson et al., 2016). This study importantly highlights the need for further research examining both peripheral and central toxicities associated with chemotherapy treatment. Interestingly, the selective serotonin reuptake inhibitor, fluoxetine, has shown promising results in a rat model of CICI. Fluoxetine co-administration with the chemotherapy drug improved cognitive performance in rats assessed by object location recognition (Lyons et al., 2012). Whilst cellular proliferation in the dentate gyrus significantly reduced in the chemotherapy group, co-administration with fluoxetine reversed this reduction. Regardless of the evidence presented here indicating CNS changes following chemotherapy exposure, it is important to note that some studies have reported no structural or cognitive changes (Fremouw et al., 2012; Wilson and Weber, 2013). Various cytotoxic insults have revealed no morphological changes to neurons located in the CNS (Ginos et al., 1987; Gangloff et al., 2005). These negative findings could result from a range of factors, including differences in species, drug, dose, type of administration and cognitive parameters examined; but importantly, suggests that more complex mechanisms are likely to play a role in CICI.

Whilst the direct mechanisms presented here reflect the complex etiology of CICI, they fail to acknowledge the influence other simultaneously occurring side-effects may be having on CICI symptoms. Many of the CICI models described above utilized chemotherapy drugs that are also often used to examine mucositis, for example 5-Fluorouracil (5-FU), methotrexate and oxaliplatin. Although mucositis would have most likely been present in these models, the gut tissue would not have been examined and thus, the potential for *indirect* mechanisms relating to gut-brain axis dysregulation would have been ignored. In doing so, we may be missing critical mechanisms contributing to or exacerbating CICI pathogenesis. In order to explore this theory, we will now consider the influence chemotherapy exposure has on the gut-brain axis, opening novel hypotheses surrounding how mucositis may contribute to the etiology of CICI.

CHEMOTHERAPY INTERRUPTS SEVERAL STAGES OF THE GUT-BRAIN AXIS

As presented above, the two organs most vulnerable to the toxicities of chemotherapy treatment are the gut and the brain. Therefore, it is plausible that several stages of the gut-brain axis may become dysregulated in the chemotherapy setting (**Figure 4**). Here, we propose that chemotherapy exposure influences the gut-brain axis via several mechanisms which include: altering intestinal microbiota composition and function; upsetting the balance of "beneficial" and "detrimental" bacteria in the lumen, deleteriously affecting the gut lining, impairing the ENS and activating neuroimmune and pain signaling pathways (**Figure 4**). The interaction of the gut-brain axis and the neuropsychological comorbidities associated with specific gut disorders have been extensively reviewed, for example depression/cognitive deficits and IBS/IBD (Whitehead et al., 2002; Attree et al., 2003; Filipovic and Filipovic, 2014; Fond et al., 2014; Padhy et al., 2015). However, this angle of research is yet to be reviewed in the context of chemotherapy exposure and cognitive impairment. Research in this area will continue to develop as we begin to appreciate that chemotherapy-induced side-effects involving the gut-brain axis may continue to linger for some time after treatment cessation, placing significant strain on health care and importantly, patient quality of life.

The Microbiome

It has been estimated that our gut contains 100-fold more genes than the human genome and approximately 1,000 bacterial species (Ley et al., 2006; Qin et al., 2010). Our gut microbiome coevolves with us (Ley et al., 2008) and changes may be either beneficial or detrimental to human health. In healthy individuals, the gut microbiota is responsible for a number of health benefits, such as pathogen protection, nutrition, host metabolism and immune modulation (O'Hara and Shanahan, 2006). Although a core microbial population has been established in individuals, changes can be caused by many factors including age, diet, antibiotic and analgesic use and environmental factors (Jalanka-Tuovinen et al., 2011). The microbiome facilitates intestinal homeostasis and more specifically, has the capacity to influence inflammation and immunity, both at the local (mucosal) and systemic levels (Clemente et al., 2012). Commensal bacteria play important roles in anti-viral immunity, regulating systemic

immune activation (Abt et al., 2012). Signals released by commensal bacteria assist in immune development and thereby, have important implications for infectious and inflammatory disease susceptibility (Ichinohe et al., 2011; Abt et al., 2012), such as in the case of chemotherapy-induced mucositis. Consequently, dysbiosis can heavily influence pathological intestinal conditions with an inflammatory component, for example in experimentally-induced IBD (García-Lafuente et al., 1997; Dalal and Chang, 2014; Touchefeu et al., 2014; Håkansson et al., 2015). Critically, IBD patients reported microbial composition changes with major shifts in genomic landscape and functional outcomes (Morgan et al., 2012). Undoubtedly, the implications of such IBD studies have heavily impacted oncology, raising many questions specifically relating to the intestinal microbiota, immune, malignancy and anti-cancer treatment interactions.

Whilst Sonis' five-phase model of mucositis (Sonis, 2004) lacked any potential influence on the microbiota, unequivocal research has indeed confirmed that intestinal inflammation modulates microbiome composition and function (Morgan et al., 2012; Touchefeu et al., 2014). As intestinal inflammation is a common characteristic of mucositis, it makes sense that chemotherapy induces functional and compositional changes to the microbiome. It has been suggested that mucositis development is influenced by commensal bacteria in multiple pathways involving inflammation and oxidative stress, intestinal permeability (discussed below), mucus layer composition, epithelial repair mechanisms and via the release of immune effector molecules (van Vliet et al., 2010). Indeed, research has begun to unravel the complexities surrounding the interactions between the host and the intestinal microbiota following chemotherapy exposure and consequently, several excellent reviews exist (van Vliet et al., 2010; Touchefeu et al., 2014; Dzutsev et al., 2015; Vanhoecke et al., 2015; Zitvogel et al., 2015). Commonly used chemotherapy drugs, such as 5-FU and irinotecan report drastic shifts in intestinal microflora, from commensal bacteria which maintain a symbiotic relationship with the host, to elevated levels of *Escherichia* spp., *Clostridium* spp., and *Enterococcus* spp. which can be associated with several pathologies involving inflammation and infection (Von Bültzingslöwen et al., 2003; Stringer et al., 2007, 2009; Lin et al., 2012; **Table 1**). Several clinical studies have supported pre-clinical findings describing alterations in fecal microbial composition following chemotherapy treatment. Literature reveals a general decrease in the overall diversity of bacteria in the microbiota of cancer patients undergoing anti-cancer treatment when compared to healthy individuals, irrespective of cancer type or chemotherapy regime (Manichanh et al., 2008; Zwielehner et al., 2011; Montassier et al., 2014; see **Table 1**).

In addition to the direct effects microorganisms and their enzymes have on cancer initiation and progression (Sears and Garrett, 2014; Gagnière et al., 2016), the microbiota also modifies drug absorption and metabolism via gene expression changes (Carmody and Turnbaugh, 2014; Wilson and Nicholson, 2017). This has become a pivotal research angle in oncology as chemotherapy-microbiota-immune interactions have identified microbial-mediated innate and adaptive immune responses and their effect on the efficacy of cancer immunotherapy and chemotherapy drugs (Iida et al., 2013; Viaud et al., 2013; Sivan et al., 2015; Vétizou et al., 2015). Two crucial studies in *Science* Iida et al. (2013); Viaud et al. (2013) reported that microbiota disruption by antibiotic treatment impaired chemotherapy drug efficacy on tumors, utilizing cyclophosphamide and oxaliplatin. More recent studies have illustrated the important role certain microbial strains (*Bifidobacterium*) play in anti-tumor immunity (Sivan et al., 2015; Vétizou et al., 2015). Although these studies were performed in mice, their findings indicate the potential risks associated with the use of antibiotics during chemotherapy treatment. The growing field of microbiome research has raised a lot of questions and comments on the complex interplay and interwoven relationships between microbes and cancer, including anti-cancer treatments (Pennisi, 2013; Bordon, 2014; Greenhill, 2014; Lokody, 2014; Mukaida, 2014). Further, some of the above studies (Sivan et al., 2015; Vétizou et al., 2015) have implications for microbial therapy in cancer immunotherapy. As our understanding of these interactions continues to progress, new knowledge in this area will open up possibilities of novel paradigm shifts in treatment approaches which may improve anticancer efficacy and even prevent toxicity. The studies presented in this section suggest a role for chemotherapy-induced dysbiosis in intestinal disease pathogenesis and chemotherapy-induced gut-brain axis dysregulation (**Figure 4**). As mentioned, commensal bacteria are critical in regulating intestinal homeostasis and more specifically, intestinal integrity. In fact, the effects commensal bacteria have on intestinal integrity and vice versa, go hand-in-hand. Accordingly, the reciprocal relationship shared between commensal bacteria and the intestinal wall will be presented together in the following section. Chemotherapy compromises intestinal integrity and leads to profound effects on the gut lining, eventually leading to a dysbiotic microbial community and consequently risking microbial invasion into the systemic circulation.

Chemotherapy Impairs Intestinal Barrier-Microbiota Interactions

The even comprehensively described pathogenesis of mucositis (Sonis, 2004) is unable to fully encapsulate the mechanisms underlying the pathogenesis of chemotherapy-induced gut damage. Although it covers many essential aspects of the pathological processes underlying mucositis, such as epithelial barrier damage. More recently, some research groups have re-defined gut damage caused by chemotherapy as chemotherapy-induced gut toxicity (CIGT). The proposed term includes additional pathological manifestations caused by chemotherapy treatment, such as abnormalities in tight junctions, immune dysfunction and microbiota influences (Montassier et al., 2014; Touchefeu et al., 2014; Wardill et al., 2014).

Nonetheless, the epithelial barrier lining of the GIT is fundamental in ensuring the maintenance of intestinal integrity. As well as forming a mechanical barrier to separate the inside of the body from the outside world, it is heavily involved in the communication shared between the body

TABLE 1 | Summary of key papers highlighting chemotherapy-microbiota-immune interactions.

Study	Subjects	Treatment	Commentary
Lin et al., 2012	Tumor bearing rats	Irinotecan alone Irinotecan/5-FU	Increased abundance *clostridial clusters I*, *XI*, and *Enterobacteriaceae*.
Von Bültzingslöwen et al., 2003	Rats	5-FU	Increased facultative and anaerobic bacteria from the oral cavity. Increased facultative anaerobes in large intestine. Proportion of facultative gram-negative rods increased in both oral cavity and intestine.
Stringer et al., 2009	Rats	Irinotecan	Increased jejunal samples of *Escherichia* spp., *Clostridium* spp., *Staphylococcus* spp. Increased colonic samples of *Escherichia spp*., *Clostridium spp*., *Enterococcus spp*., *Serratia spp*., *Staphylococcus spp*. No changes in fecal flora except *E. coli*.
Stringer et al., 2007	Rats	Irinotecan	Extensive changes were evident in stomach, jejunum, colon and feces. Most significant changes were in colon, indicating a relationship between colon bacteria modification and diarrhea incidence.
Montassier et al., 2014	Patients with non-Hodgkin's lymphoma	Bone marrow transplantation with chemotherapy conditioning	Steep reduction in alpha diversity during chemotherapy. Decreases in *Firmicutes bacteria* and *Bifidobacterium* whilst *Bacteriodes* and *Esterichia* were increased
Manichanh et al., 2008	Patients with abdominal tumors	Pelvic radiotherapy	Faecal samples reported significant microbiota profile changes in patients with post-radiotherapy diarrhea. Not all patients reported diarrhea. Importantly, this study suggests initial microbial colonization may be linked to susceptibility or protection against diarrhea following radiotherapy treatment.
Zwielehner et al., 2011	Patients with various malignancies	Chemotherapy and antibiotic treatment	Chemotherapy decreased *Clostridium cluster IV* and *XIVa*. *C. difficile* was present in three out of seventeen patients and was accompanied by a decrease in the genera *Bifidobacterium*, *Lactobacillus*, *Veillonella* and *Faecalibacterium prausnitzii*. *Enterococcus faecium* increased following chemotherapy.
Iida et al., 2013	Tumor bearing mice	Oxaliplatin and cisplatin	Chemotherapy-induced dysbiosis impairs response to immunotherapy and chemotherapy.
Viaud et al., 2013	Tumor bearing mice	Cyclophosphamide	Jejunal and fecal samples reported dysbiosis and induces translocation of specific Gram-positive bacteria to secondary lymphoid organs whereby they stimulate subsets of T cells. These results suggest that the gut microbiota may affect anticancer immune response.
Sivan et al., 2015	Tumor bearing mice	Co-housing, fecal transfer, programmed cell death protein 1 ligand 1 (PD-L1)–specific antibody therapy (checkpoint blockade), oral *Bifidobacterium*	Changes to anti-tumor immunity were eliminated by co-housing and fecal transfer. Oral *Bifidobacterium* administration improved tumor control to same degree as PDL-1 therapy; combination treatment nearly abolished tumor outgrowth.
Vétizou et al., 2015	Tumor bearing mice and metatstatic melanoma patients	Ipilimumab (CTLA-4 blocker) regulates T cell activation and improves survivability of metastatic melanoma patients.	CTLA-4 blockade is influenced by the microbiota. Changes in *B. fragilis* and/or *B. thetaiotaomicron* and *Burkholderiales* affects immune response facilitating tumor control in mice and patients.

and the intestinal contents (Powell, 1981). Tight junctions are intertwined throughout the epithelial barrier and regulate diffusion of solutes according to strict size and charge limitations (Balda and Matter, 2016). Chemotherapy exposure increases intestinal permeability and the most widely studied mechanisms to date have included apoptosis of intestinal crypts and villous atrophy (Keefe et al., 2000; Carneiro-Filho et al., 2004). Early clinical studies assessing the severity of intestinal damage in high dose regimes reported abnormalities in intestinal permeability and defects in tight-junction integrity (**Figure 4**; Keefe et al., 1997). Convincing rodent evidence has implicated mucosal barrier injury and tight junction deficits with gut toxicity induced by various chemotherapy drugs, including irinotecan and methotrexate (Beutheu Youmba et al., 2012; Wardill et al., 2014). However, it should be noted that rodent model application in gut immunity and microbiome research has serious limitations

and pitfalls due to compositional microbiota differences between species. Whilst the rodent microbiome shares some common features with human commensal bacteria, unique commensals in rodents have differential effects in immune responses and disease pathogenesis (Nguyen et al., 2015). Consequently, animal models of inflammation are different to human models of inflammation in terms of microbial colonization, morphology of lesions and clinical manifestations. Nonetheless, research in experimental models continues to provide critical insight into complex interactions between the host, microbiota and immune responses. More recently, it has been becoming more evident the impact intestinal integrity has on the microbiota and vice versa, especially under chemotherapy conditions. The health and stability of the intestinal wall influences the microbiota and vice versa.

Commensal bacteria in the microbiota have a protective effect on intestinal integrity, interacting with TLR and Nuclear Factor kappa B pathways, ensuring the development of an innate immune response (Doyle and O'Neill, 2006). These components of innate immunity in the gut and the activation of these pathways are pivotal in maintaining barrier function, promoting mucosal repair and protecting the gut against injury (Rakoff-Nahoum et al., 2004; Cario, 2008). Chemotherapy exposure alters commensal microbial composition in the microbiota, thus negatively affecting barrier function, repair pathways and compromising intestinal integrity (Stringer et al., 2009). Accordingly, further investigations are required to fully appreciate the role chemotherapy-induced intestinal permeability changes play in gut-brain axis dysregulation. As intestinal integrity becomes compromised under chemotherapy conditions, it is not surprising that nerves of the myenteric plexus and peripheral nerve endings become damaged as these neural components also reside outside of the blood-brain barrier (BBB) as will be discussed below.

Chemotherapy Results in Peripheral and Enteric Neuropathy

The PNS is particularly vulnerable to the cytotoxic nature of different chemotherapy drug classes, including platinum analogs, antitubulins, proteasome inhibitors, immunomodulatory agents and some newer biologics, such as brentuximab (Cavaletti and Marmiroli, 2015). Chemotherapy-induced peripheral neuropathy (CIPN) is experienced by 30–40% of chemotherapy recipients and is often responsible for early cessation of treatment, decreasing chemotherapeutic efficacy and causing higher relapse (Wang et al., 2012; Areti et al., 2014). Typically, sensorimotor symptoms are more common than motor involvement, presenting in a bilateral "glove-and-stocking" distribution in the hands and feet to include paraesthesia, numbness, burning pain, allodynia and hyperalgesia (Windebank and Grisold, 2008). However, the development of motor and autonomic neuropathic symptoms may also occur, such as sensory ataxia, pain, weakness of distal muscles, reduced deep tendon reflexes and severe numbness that can severely affect the patient's ability to function and their quality of life (Park et al., 2013). Often symptoms fail to improve after cessation of treatment, referred to as a "coasting" phenomenon (Windebank and Grisold, 2008).

The pathogenesis of CIPN is primarily related to axonopathy and neuronopathy in which dorsal root ganglia (DRG) are involved. Peripheral nerves and their ganglia are particularly susceptible to chemotherapy-induced damage due to their location as they lack the protective defenses associated with the BBB (Furness et al., 2014). For example, chemotherapy interrupts the cell cycle, inducing structural and functional changes in DRG which partly explain the development of sensory symptoms in CIPN (Gill and Windebank, 1998; Cavaletti et al., 2000). Many pathophysiological mechanisms mediating chemotherapy-induced peripheral nerve damage have been identified. Some examples include, but are not exclusive to dysregulated axonal transport and trophic factor support via microtubule structural changes (Theiss and Meller, 2000), mitochondrial stress (McDonald and Windebank, 2002; Chen et al., 2007) and reduced blood supply to nerves (Theiss and Meller, 2000; Isoardo et al., 2004). Further changes contributing to CIPN pathogenesis include dysregulated ion channels, neurotransmitter release and receptor sensitivity (Descoeur et al., 2011; Mihara et al., 2011; Tatsushima et al., 2011). The evidence presented here clearly describes mechanisms by which the PNS is damaged following chemotherapy exposure, forming an important element of the proposed central hypothesis (**Figure 4**).

In addition to peripheral neuropathies, neurons residing within the ENS are also susceptible to the deleterious effects of various chemotherapy drugs, including cisplatin, oxaliplatin and more recently, 5-FU (Vera et al., 2011; Wafai et al., 2013; McQuade et al., 2016). Systemic administration of these chemotherapy drugs induces structural and functional changes to myenteric neurons (**Figure 4**), consequently resulting in downstream negative effects on GI motility. Interestingly, acute exposure of 5-FU increases intestinal transit whilst prolonged treatment decreases transit time (McQuade et al., 2016). These findings outline the complex nature chemotherapy drugs have on enteric neurons and altered motility patterns. Here, we highlight that chemotherapy results in damage to neurons and ganglia residing outside of the BBB, exerting functional maladaptations in both the PNS and ENS. So far we have described several mechanisms relating to chemotherapy-induced gut-brain axis dysregulation, yet we have not identified how immune signals from the intestinal cavity may communicate to the brain and potentially contribute to the pathogenesis of CICI. In the following section we present peripheral-to-central immune pathways as being critical in the transmission of signals from the gut to the brain following chemotherapy exposure.

Peripheral-to-Central Immune Signaling Pathways Mediating Chemotherapy-Induced Gut-Brain Axis Dysregulation

Historically there has been controversy surrounding the theory that a communication system existed between the immune system and the CNS (Dantzer and Kelley, 2007). Traditionally

it was assumed that proinflammatory cytokines were unable to pass through the BBB due to their size. However, the humoral route explained that cytokines expressed in the periphery could in fact cross the BBB at leaky circumventricular organs through fenestrated capillaries. At these sites blood-borne cytokines act on parenchymal astrocytes that express secondary mediators, such as nitric oxide and prostaglandins which freely diffuse to nearby brain regions, such as the hypothalamus to mediate the effects of pyrogenic and corticotropic cytokines (Katsuura et al., 1990). Whilst this hypothesis leads toward the existence of a communication system between the immune system and the CNS, it was unable to fully account for other contributing pathways that may be mediating physiological responses. Consequently, it is now widely accepted that peripheral cytokines signal the brain and in turn, this triggers sickness behavior responses (Dantzer, 2004).

Central or peripheral immune challenges trigger a range of physiological, behavioral and motivational changes to assist the host in healing (**Figures 2**, **4**). Non-specific symptoms which accompany sickness behaviors include, but are not exclusive to fever, depressed activity, a loss of interest in regular activities (appetite, sexual, cleaning, hygiene), weakness, malaise, listlessness and cognitive changes (Dantzer and Kelley, 2007). As demonstrated by Dantzer and Kelley (Dantzer and Kelley, 2007), the last two decades of research on this phenomenon have confirmed that local or systemic proinflammatory cytokines expressed at physiological levels, during both acute and chronic inflammatory responses, serve as true communication molecules between the immune system and brain. For example, direct administration of IL-1β or TNF-α to the lateral ventricle decreased social exploration and feeding behavior in rats (Kent et al., 1992). In the chemotherapy setting, this phenomenon may be related to either the systemic nature of the drugs themselves or localized inflammatory responses occurring as a result of the toxicities associated with their use, such as in the case of mucositis (see **Figure 2**).

Interestingly, whilst IL-1β and TNF-α are key proinflammatory cytokines instigating sickness behavior responses, they also play a pivotal role in the pathogenesis of mucositis. Since both proinflammatory cytokines play an important role in the pathogenesis of mucositis and sickness behaviors which involve cognitive deficits, it is plausible that these cytokines and the pathways mediating their activation may present as key mechanisms underlying the central hypothesis in this review (**Figure 4**). Various animal models have identified that sickness behavior responses may be induced by a range of clinical conditions, such as systemic or central administration of lipopolysaccharide (active fragment of gram negative bacteria) or recombinant proinflammatory cytokines (Tomas et al., 1984; Goehler et al., 1999; Dantzer and Kelley, 2007). Furthermore, many symptoms associated with cytokine-induced sickness responses mimic the cluster of chemotherapy-induced side-effects, including fatigue, depression, reduced appetite, heightened sensitivity to pain and cognitive impairment (**Figure 4**). As previously mentioned, up to 70% of chemotherapy recipients experience mucositis (Scully et al., 2003) and an altered immune profile due to the systemic nature of anti-cancer treatments, yet whether these side-effects may be contributing to CICI remains elusive. Accordingly, we present pathways which may be enabling the communication of peripheral immune signals to the brain, more specifically defining how mucositis-driven inflammation may signal the brain via vagal- and neural-mediated mechanisms and contribute to the pathogenesis of CICI.

Information from proinflammatory cytokines and mediators expressed under chemotherapy-induced mucositis conditions may signal the brain via a vagal communication pathway (**Figure 4**). Dendritic cells (DCs) are a specialized subset of immune cells located within the vagus nerve and surrounding paraganglia (Goehler et al., 1999). The signals (proinflammatory cytokines, chemokines and mediators) expressed by DCs are capable of communicating to the brain (Banchereau and Steinman, 1998; Reis e Sousa et al., 1999). Vagal immunosensation requires primary afferent neuron activation as the initial interface triggering the brain. Following chemotherapy exposure, proinflammatory cytokines and mediators, such as those from the IL-1 family arise from mucositis-induced inflammation. IL-1 binds to receptors on the paraganglia surrounding vagal afferents and release neurotransmitters onto the vagus, consequently activating vagal fibers. A vagal-mediated neural signal is then carried to the nucleus tractus solitarius which projects the message to higher order brain regions, such as the hypothalamus and hippocampus, whereby, IL-1 production is increased and other neural cascading events are initiated to produce sickness behavior responses (Dantzer and Kelley, 2007; Wardill et al., 2015). Whilst this evidence clearly demonstrates the role that vagal afferent nerves play in peripheral-to-central transmission of immune messages from the abdominal cavity, to date these pathways have not been examined under chemotherapy conditions and are therefore presented as potential mechanisms contributing to gut-brain axis dysregulation.

DCs play a role in immunomodulation and neuroimmune regulation, crucially bridging innate and immune adaptive processes. Importantly, DCs express pattern recognition receptors for a range of chemicals (e.g., TLRs), chemokines, microorganisms and neurotransmitters, such as serotonin (Banchereau and Steinman, 1998; Reis e Sousa et al., 1999). Damage to surroundings GIT tissues and increased levels of proinflammatory mediators, such as cytokines and chemokines induce maturation of DCs (Ricart et al., 2011). Matured DCs migrate to secondary lymphoid organs to initiate a localized immune response via interacting with naïve T cells (Banchereau and Steinman, 1998). Recent data implies an emerging role for DC-expressed serotonin and receptor activation in regulating innate and immune responses associated with gut inflammatory conditions (Holst et al., 2015; Szabo et al., 2018). Mechanisms underlying DC-mediated serotonin and receptor-sub types affect various levels of localized inflammation, even having anti-inflammatory effects preventing excess inflammation and tissue damage (Szabo et al., 2018). These findings coupled with the aforementioned positive cognitive effects of fluoxetine in a rat model of CICI (Lyons et al., 2012), serotonin presents as a new therapeutic approach for inflammatory disorders, having effects

in both the gut and the brain. Accordingly, serotonin is presented as an underestimated contributing factor potentially implicated in chemotherapy mediated gut-brain axis dysregulation (see Wigmore, 2012).

CONCLUSION

From the bottom-up, the gut and the brain are the two primary organs most susceptible to toxicity associated with the non-selective nature of chemotherapy drugs. As chemotherapy exposure induces cognitive decline and mucositis in a subset of recipients, it makes sense that several stages of the gut-brain axis are prone to negative effects in this setting. The gut-brain axis is largely responsible for the maintenance of homeostasis and achieves this delicate balance by integrating a vast array of signals and information from many systems, as described above and shown in the figures. In this regard, upsetting the balance of any stage in the gut-brain axis following chemotherapy treatment has the potential to exacerbate side-effects, such as in the case of mucositis and CICI. The findings from our review support our main hypotheses that chemotherapy treatment causes severe and prolonged psychosocial impacts on the survivor. Secondly, the gut-brain axis is an important mediator of a diverse range of cognitive and emotional disorders similar to those experienced by cancer survivors. Evidently, chemotherapy affects the gut-brain axis at several key stages which are outlined above. Collectively, we conclude that the psycho-social side-effects of chemotherapy treatment may be caused by the effects of chemotherapy on the gut-brain axis.

Apart from chemotherapy treatments crossing the BBB and *directly* causing damage to specific regions, peripheral inflammatory responses from either the malignancy or systemic treatment also *indirectly* cause cellular changes in the spinal cord. We recently demonstrated glial dysregulation in the thoracic region of rats with 5-FU-induced intestinal mucositis indicating an indirect regional-specific neuroimmune response to CIGT (Bajic et al., 2015). Our data provides evidence that experimentally-induced jejunal toxicity *indirectly* downregulates thoracic astrocytic expression. In addition to this recent finding, the evidence presented here suggests a role for chemotherapy-induced dysbiosis in intestinal inflammation. This further complicates intestinal inflammation and ulceration induced by chemotherapy exposure which may potentially influence CICI. Neurons in both the ENS and the PNS are also vulnerable to the cytotoxic nature of chemotherapy treatments. The implications of co-administration of pharmacological interventions (e.g., fluoxetine) with chemotherapy drugs remains undetermined, although preliminary studies showing improvements in cognitive performance warrants further investigation. In view of the aforementioned data, we conclude that several stages of the gut-brain axis become dysregulated following chemotherapy exposure and may be implicated in the pathogenesis of CICI. Harnessing our understanding of the role gut-brain axis dysregulation plays in modulating brain function may offer clues for more targeted therapeutic strategies to prevent CICI and warrants further investigation.

AUTHOR CONTRIBUTIONS

JB wrote this manuscript to form part of her thesis and it is her second review. IJ, GH, and MH contributed equally at various points throughout the course of this manuscript, adding valuable input and critically reviewing each draft made by JB.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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The Role of the Intestinal Microbiome in Chronic Psychosocial Stress-Induced Pathologies in Male Mice

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Chronic psychosocial stress is a risk factor for the development of physical and mental disorders accompanied or driven by an activated immune system. Given that chronic stress-induced systemic immune activation is lacking in germ-free and antibiotics-treated mice, a causal role of the gut microbiome in the development of stress-related disorders is likely. To address this hypothesis in the current study we employed the chronic subordinate colony housing (CSC, 19 days) paradigm, a pre-clinically validated mouse model for chronic psychosocial stress, known to alter the gut microbial signature and to induce systemic low-grade inflammation, as well as physical and mental abnormalities. In detail, we investigated if (i) CSC-induced alterations can be prevented by repeated transplantation of feces (FT) from non-stressed single-housed control (SHC) mice during CSC exposure, and (ii) if the transplantation of a "stressed" CSC microbiome is able to induce CSC effects in SHC mice. Therefore, we repeatedly infused SHC and CSC recipient mice rectally with SHC donor feces at days 4 and 11 of the CSC paradigm and assessed anxiety-related behavior on day 19 as well as physiological, immunological, and bone parameters on day 20. Furthermore, SHC and CSC recipient mice were infused with CSC donor feces at respective days. To exclude effects of rectal infusions *per se*, another set of SHC and CSC mice was infused with saline, respectively. Our results showed that transplantation of SHC feces had mild stress-protective effects, indicated by an amelioration of CSC-induced thymus atrophy, anxiety, systemic low-grade inflammation, and alterations in bone homeostasis. Moreover, transplantation of CSC feces slightly aggravated CSC-induced systemic low-grade inflammation and alterations in bone homeostasis in SHC and/or CSC animals. In conclusion, our data provide evidence for a role of the host's microbiome in many, but not all, adverse consequences of chronic psychosocial stress. Moreover, our data are consistent with the hypothesis that transplantation of healthy feces might be a useful tool to prevent/treat different adverse outcomes of chronic stress. Finally, our data suggests that stress effects can be transferred to a certain extend via FT, proposing therapeutic approaches using FT to carefully screen fecal donors for their stress/trauma history.

Keywords: chronic psychosocial stress, chronic subordinate colony housing (CSC), anxiety, bone homeostasis, inflammation, microbiome, fecal transplantation

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INTRODUCTION

Chronic psychosocial stress is an acknowledged risk factor for the development of various somatic and affective disorders, including inflammatory bowel disease (IBD) and posttraumatic stress disorder (PTSD) (Duffy et al., 1991; Agid et al., 1999; Bitton et al., 2003; de Kloet et al., 2005; Bernstein et al., 2010; Virtanen and Kivimaki, 2012; Virtanen et al., 2012; Langgartner et al., 2015; Reber et al., 2016a,b). However, although chronic stress results in a dysregulation of almost all neuro-immuno-endocrine systems of an organism, the exact mechanisms underlying these stress-related disorders are still largely unknown. A relatively novel, but promising, hypothesis in this context is that the pathogenic potential of chronic stress is, at least in part, mediated by the intestinal microbiome (Mackos et al., 2015, 2017; Tarr et al., 2015; Galley et al., 2017; Lafuse et al., 2017).

According to recent findings, the ratio of bacterial to eukaryotic cells in the human body is approximately 1:1 (Sender et al., 2016), resulting in an overall microbial mass comparable to the weight of the human brain (Stilling et al., 2014, 2016). The majority of these microorganisms resides in the gut (Sender et al., 2016), comprising around 150 times more genes than the human genome (Qin et al., 2010). Thus, it is not surprising that the gut microbiome is able to influence the host in a variety of parameters. For instance, rodent studies have shown that the transfer of donor microbiome strongly affects recipients' behavior, physiology and metabolism (Bercik et al., 2011; Collins et al., 2013). Furthermore, fecal transplantation (FT) from depressed patients into rats pretreated with antibiotics induces depressive-like behavior (Kelly et al., 2016) and colonizing germ-free (GF) mice with the gut microbiota of IBD patients causes barrier dysfunction and innate immune activation (De Palma et al., 2017). Moreover, colonization of GF mice with a specific-pathogen free (SPF) microbiome promotes an increased bone formation and resorption (Yan et al., 2016).

In turn, factors like diet (Singh et al., 2017), age (Mariat et al., 2009), pharmaceutics (Langdon et al., 2016), but also stressor exposure are well-known to affect the gut microbiome, although the latter is very stable over time (Allison and Martiny, 2008). For instance, PTSD in humans (Hemmings et al., 2017) and psychological stressors in rodents (O'Mahony et al., 2009; Bailey et al., 2010, 2011; Cui et al., 2016) are associated with compositional changes of the gut microbiome, paralleled by immune activation as indexed by increased circulating interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 (Bailey et al., 2011) levels. Of note, these bidirectional processes between the host and the microbiome are referred to as the "microbiome-gut-brain-axis" (MGBA) and involve different neuro-immuno-endocrine mediators, including the vagus nerve, stress hormones, the immune system as well as the tryptophan metabolism and microbial metabolites (Cryan and Dinan, 2012).

The "chronic subordinate colony housing" (CSC) paradigm is a well-characterized animal model for PTSD in male mice (Reber et al., 2016a) and causes chronic low-grade inflammation (Langgartner et al., 2018), disturbance of bone homeostasis (Foertsch et al., 2017), general and social anxiety (Slattery et al., 2012; Langgartner et al., 2017), and, in the presence of colonic pathobionts like *Helicobacter* spp. (Langgartner et al., 2017), spontaneous colitis (Reber et al., 2007, 2011, 2016b; Langgartner et al., 2017). Although CSC further causes an increase in β-diversity (microbial diversity between different samples) and a decrease in α-diversity (microbial diversity within one sample) within the gut microbiome (Reber et al., 2016a,b), as described for many chronic stressors (Bailey, 2014), it is not known whether these compositional changes are critically involved in the above described behavioral, physiological, and immunological consequences of CSC.

Therefore, we in the current study aimed to test in a first approach if repeated transfer of a healthy SPF gut microbiome from non-stressed single-housed control (SHC) mice into CSC mice using FT is able to prevent CSC-induced behavioral, physiological, and immunological consequences. In a second approach, we also tested if transplantation of a "stressed" CSC microbiome is able to induce well-known CSC effects in SHC mice.

MATERIALS AND METHODS

Animals

Male C57BL/6N (weighing 19–22 g) mice were used as both fecal donor and fecal recipient animals and male CD-1 mice (weighing 30–35 g) were used as dominant residents. All animals were obtained from Charles River (Sulzfeld, Germany). After delivery, donor as well as recipient mice were individually housed in a SPF animal facility under a 12 h/12 h light dark cycle, 22°C, 60% humidity, and had free access to tap water and standard mouse diet. The animal study was carried out in accordance with the relevant guidelines and regulations and was approved by the Federal Animal Care and Use Committee [Regierungspraesidium Tuebingen, Germany (permit No.: 1195)].

Experimental Procedures

One to two weeks after arrival, mice were either exposed to the CSC paradigm (days 1–20) or kept as SHC mice (days 1–20; for details, see section "Chronic Subordinate Colony Housing (CSC) Procedure"). All experimental mice were weighed on days −6, 1, 4, 8, 11, 15, 19, and 20. Three sets of CSC and SHC animals were used in this study. In detail, on days 4 and 11, animal set 1 (SHC: n = 20, CSC: n = 16) was infused rectally with saline, animal set 2 (SHC: n = 12, CSC: n = 12) with SHC donor feces, and animal set 3 (SHC: n = 12, CSC: n = 12) with CSC donor feces. All animals were tested for anxiety-related behavior in the open-field/novel object (OF/NO) test on day 19 of CSC between 07:00 and 10:00 AM, and sacrificed in the morning of day 20 between 07:00 and 10:00 AM following brief CO_2 anesthesia. Afterward, absolute adrenal- and thymus weight, plasma corticosterone (CORT), CORT production from *in vitro* adrenocorticotropic hormone (ACTH)-stimulated adrenal explants, and the histological damage score were assessed in all these mice. In addition, local [mRNA expression of colonic tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), cathelin-related antimicrobial peptide (CRAMP), colonic protein

expression of F4/80 and cluster of differentiation molecule (CD11b)] and systemic [keratinocyte chemoattractant (KC), IL-6, and monocyte chemotactic protein-1 (MCP-1)] inflammatory parameters were measured in some mice of each animal set [local: Set 1 (SHC: $n = 8$, CSC: $n = 4$); Set 2 (SHC: $n = 12$, CSC: $n = 12$); Set 3 (SHC: $n = 12$, CSC: $n = 12$); systemic: Set 1 (SHC: $n = 8$, CSC: $n = 4$); Set 2 (SHC: $n = 4$, CSC: $n = 4$); Set 3 (SHC: $n = 8$, CSC: $n = 4$)]. Moreover, bone homeostasis [tibia length, femoral growth-plate width, trabecular thickness, trabecular bone mineral density (BMD), trabecular bone volume/tissue volume (BV/TV) and trabecular number] parameters were assessed in some mice of each animal set [Set 1 (SHC: $n = 8$, CSC: $n = 4$); Set 2 (SHC: $n = 4$, CSC: $n = 4$); Set 3 (SHC: $n = 8$, CSC: $n = 8$)]. Two animals (CSC-saline) died due to unknown reasons before day 19, and thus were excluded from all parameters. One animal (CSC-saline) died due to unknown reasons between d19 and d20, and thus was excluded from the physiological parameters. Two adrenal glands (CSC-saline) were injured during the preparation procedure and thus excluded from the analysis. Of note, donor mice were exposed to either SHC or CSC exposure, starting on the same day as SHC or CSC exposure of respective recipient mice. In other words, feces infused on days 4 and 11 into recipient mice originated from donor mice exposed to SHC or CSC for either 4 or 11 days, respectively. Donor feces were collected from overall $n = 28$ SHC and $n = 24$ CSC mice. For more details, see section "Preparation of Fecal Suspension and Transplantation Procedure."

Chronic Subordinate Colony Housing (CSC) Procedure

The CSC procedure was performed as described in previous publications (Reber et al., 2007; Langgartner et al., 2015). Briefly, experimental mice were weighed and assigned to either the SHC or the CSC group, matched according to their body weight. The bedding of SHC mice was changed once a week. In order to induce chronic psychosocial stress, a group of four CSC mice was housed together with a dominant male CD-1 mouse for 19 consecutive days. To avoid habituation, CSC mice were placed into the home cage of a novel dominant male CD-1 mouse on days 8 and 15. All mice were weighed twice a week and received a rectal infusion of either saline (animal set 1), SHC donor feces (animal set 2) or CSC donor feces (animal set 3) on days 4 and 11 of CSC, respectively.

Preparation of Fecal Suspension and Transplantation Procedure

To collect feces from donor animals, mice of both the SHC and CSC donor group were housed individually (without bedding) during stool pellet collection (approximately 15–20 min; 2–3 stool pellets per mouse). Collected and pooled (per group) fecal pellets were stored on ice (4°C) until homogenization in isotonic saline solution (1 part feces + 4 parts saline; Fresenius Kabi, Bad Homburg, Germany) using the Vortex Genie 2 (Scientific Industries Inc., Bohemia, NY, United States). To prevent congesting of the feeding needle (Heidelberg, Germany), which was used to rectally infuse the fecal suspension, the homogenate was filtered through a cell strainer (70 μm, Corning, Durham, NC, United States) to remove coarse particles. Subsequently (between 08:00 and 10:00 AM), recipient mice were rectally infused with 100 μl of the respective solution [saline (animal set 1), SHC donor feces (animal set 2) or CSC donor feces (animal set 3)].

Open-Field/Novel Object Test

To assess CSC effects on anxiety-related behavior, SHC and CSC animals were exposed to the OF/NO test on day 19 of CSC exposure. Briefly, the arena (45 cm length × 27 cm width × 27 cm height) was subdivided into an inner (27 cm × 9 cm) and an outer zone. The arena was cleaned thoroughly before each trial. Within each trial, the mouse was placed into the inner zone and was allowed to explore the arena for 5 min. After 5 min of open-field exploration, a plastic round object (diameter: 3.5 cm; height: 1.5 cm) was placed in the middle of the inner zone. The mouse now was allowed to explore the arena containing the unfamiliar object for 5 min. In the open-field test, the number of inner zone entries and the time spent in the corners of the arena (measurements of anxiety-related behavior) as well as the distance moved (measurement of general activity) were assessed. In the novel object test, the number of object explorations and the time spent in the corners of the arena as well as the distance moved (all parameters of anxiety-related behavior) were analyzed. All parameters were analyzed using EthoVision XT (Version 9, Noldus Information Technology, Wageningen, Netherlands). The test was performed between 07:00 and 10:00 AM under white light conditions (350 lux).

Trunk Blood Sampling

All mice were euthanized by decapitation in the morning of day 20. In detail, within 3 min after removing the cage from the animal room, mice were rapidly euthanized by decapitation following brief CO_2-exposure. Trunk blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes (Sarstedt, Nuembrecht, Germany) and stored on ice until centrifugation. Tubes were centrifuged at 4°C (5,000 × *g*, 10 min). Plasma samples were stored at −20°C until further analysis.

Assessment of Adrenal- and Thymus Weight

Adrenal glands were removed, pruned of fat, weighed, and stored in ice-cold Dulbecco's Modified Eagle Medium [DMEM/F-12, Life Technologies, Inc., Grand Island, NY, United States; supplemented with 0.1% bovine serum albumin (BSA; Biomol, Hamburg, Germany)], until used for *in vitro* ACTH stimulation. Thymus was removed and stored in ice-cold phosphate buffered saline (PBS; Life Technologies) until all animals were euthanized. Subsequently, thymus glands were pruned of fat and weighed separately.

In vitro ACTH Stimulation of Adrenal Explants

Adrenal *in vitro* ACTH stimulation was performed as described previously (Uschold-Schmidt et al., 2012). Briefly, after pruning

from fat and weighing, adrenals were stored in ice-cold DMEM (DMEM/F-12, Life Technologies, Inc.) supplemented with 0.1% BSA, until further treatment. Adrenal glands were then cut into two halves (with each containing a cortical and medullary part), weighed again and pre-incubated in 200 µl DMEM/F-12 for 4 h (37°C, 5% CO_2). Afterward, culture medium was replaced by fresh medium, thereby each half of one adrenal gland was incubated with either medium containing saline (basal) or medium containing ACTH (100 nM; representing a pharmacological dose of ACTH) for 6 h (37°C, 5% CO_2). After 6 h of stimulation, supernatants were carefully removed and stored at −20°C until further analysis. Samples were analyzed using a commercially available ELISA for CORT (analytical sensitivity: <0.564 ng/ml, intra-assay and inter-assay coefficients of variation ≤6.35%; IBL International, Hamburg, Germany). CORT concentrations were calculated in relation to the weight of the respective adrenal explant.

Enzyme-Linked Immunosorbent Assay (ELISA) for Plasma CORT and Multiplex Cytokine Analysis

Plasma samples were analyzed using a commercially available ELISA for CORT (analytical sensitivity: <0.564 ng/ml, intra-assay and inter-assay coefficients of variation ≤6.35%; IBL International, Hamburg, Germany). Further, plasma concentrations of KC, IL-6 and MCP-1, were determined using a mouse multiplex cytokine kit (ProcartaPlex, eBioscience, Frankfurt, Germany). Data analysis was performed using the Luminex® 100 Total System (Bio-Rad Laboratories, Hercules, CA, United States).

Quantitative Real-Time PCR Analysis of TNFα, IFNγ, and CRAMP mRNA in Colonic Tissue

In order to determine CSC and/or FT effects on colonic TNFα, IFNγ, and CRAMP mRNA expression, one piece of colonic tissue was frozen at −80°C until starting the RNA isolation. RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions, including DNase I (Qiagen, Valencia, CA, United States) digestion. The amount of RNA was measured using a Nanodrop 2000 (Thermo Fischer Scientific, Waltham, MA, United States). 1 µg of RNA was used to generate cDNA employing random primers (High-capacity cDNA reverse transcription kit, Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, United States) and additional RNase inhibitor "RNase out" to reduce RNase activity (Invitrogen, Thermo Fischer Scientific, Waltham, MA, United States). Real Time PCR was performed using a ViiA7 Real-Time PCR System (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, United States) using Platinum SYBR Green (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States). The primers (Sigma-Aldrich, St. Louis, MO, United States) used were Ribosomal Proteins (RPL; Housekeeper) (forward: 5′ CCTGCTGCTCTCAAGGTT 3′; reverse: 5′ TGGCTGTCACTGCCTGGTACTT 3′), TNFα (forward: 5′AGGGGCCACCACGCTCTTCT3′, reverse: 5′TGAGTGTGAGGGTCTGGGCCAT3′), CRAMP (forward: 5′CAGCCCTTTCGGTTCAAGAA3′, reverse: 5′CCCACCTTTGCGGAGAAGT3′) and IFNγ (forward: 5′TGCTGATGGGAGGAGATGTCT3′, reverse 5′TGCTGTC TGGCCTGCTGTTA3′). For the quantification of results, the corresponding Applied Biosystems Software was used and ΔΔ Ct analysis was performed.

Protein Analysis of F4/80 and CD11b in Colonic Tissue

To measure colonic protein expression of F4/80 and CD11b, one piece of colonic tissue was frozen at −80°C until further processing. Protein extraction was performed by homogenizing the tissue in ethylenediaminetetraacetic acid (EDTA) lysis buffer [50 mM EDTA, 250 mM NaCl, 0.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.5% Igepal, 10% Complete Mini Protease Inhibitor (Roche Diagnostics GmbH, Mannheim, Germany)]. Total protein concentration was determined using a commercially available detection kit (Bicinchoninic Acid Protein Assay Kit, Thermo Scientific, Rockford, IL, United States). Western blot analysis was performed by loading 30 µg of protein per colonic sample onto sodium dodecyl sulfate polyacrylamide gels (10%) and subsequent transfer on nitrocellulose membranes (Amersham Protran Premium 0.45 µm NC, GE Healthcare Life science, Freiburg, Germany). Membranes were then blocked for 1 h at room temperature (RT) in 5% milk powder (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) diluted in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST; VWR, Darmstadt, Germany), and afterward incubated with primary F4/80 antibody (1:1000, Santa Cruz Biotechnology, Dallas, TX, United States). overnight at 4°C. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:2000, Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany) was used as secondary antibody (1 h, RT). After incubation with ECL Western Blotting Detection Reagents (GE Healthcare, Freiburg, Germany), chemiluminescence was digitalized and analyzed using Molecular Imager® ChemiDoc™ XRS+ system and Image Lab™ (Bio-Rad Laboratories GmbH, Munich, Germany), respectively. After that, membranes were stripped using Re-Blot Plus Strong Antibody Stripping Solution (Millipore GmbH, Schwalbach, Germany), blocked twice and incubated with primary β-Tubulin (1:1000, 1 h at RT; Cell Signaling Technology, Danvers, MA, United States). HRP-conjugated anti-rabbit antibody (1:1000) was again used as secondary antibody (30 min, RT). Following visualization and digitalization, membranes were again stripped and this time incubated with CD11b antibody (1:2000; Abcam®, Cambridge, United Kingdom) over night at 4°C. Following incubation with HRP-conjugated anti-rabbit antibody (1:2000), visualization and digitalization were again performed as described above. Bands were detected at 160 kDa (F4/80), 170 kDa (CD11b), 55 kDa β-Tubulin as described by the manufacturers. The expression of F4/80 and CD11b was normalized to the respective β-tubulin protein expression.

Determination of the Histological Damage Score of the Colon

To assess CSC effects on the histological damage of the colon, the colon was removed after decapitation and cleaned. Histological damage score was assessed as described previously (Reber et al., 2007) with slight modifications. One centimeter of the distal part of the distal third of the colon was cut longitudinally and fixed in 5% formalin for 48 h. Fixed tissue was then embedded in paraffin and cut longitudinally. Two 3 μm hematoxylin–eosin stained sections taken at 100 μm distance were evaluated by histological scoring performed by an investigator blind to treatment. Each individual score represented the mean of the sections. Histological damage score ranges from 0 to 8 and represents the sum of the epithelium score (0: normal morphology; 1: loss of goblet cells; 2: loss of goblet cells in large areas; 3: loss of crypts; 4: loss of crypts in large areas) and infiltration score (0: no infiltration; 1: infiltrate around crypt bases; 2: infiltrate reaching to lamina muscularis mucosae; 3: extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; 4: infiltration of the lamina submucosa).

Assessment of Bone Homeostasis

Both femurs and right tibia were removed for further analysis and stored in 4% formalin. Tibia lengths were assessed using a digital precision caliper. Left femurs were subjected to μCT analysis as described previously (Foertsch et al., 2017). Briefly, scanning was done at 50 kV and 200 mA, voxel resolution was set to 8 μm. Analysis was conducted according to ASBMR guidelines (Bouxsein et al., 2010). BMD was assessed using two phantoms with defined hydroxyapatite (HA) contents (250 and 750 mg/cm^3). The threshold for mineralized tissue was set at 390 mg HA/cm^3 for trabecular bone. Right femurs were subjected to decalcified histology as described previously (Haffner-Luntzer et al., 2014). Sections of 7 μm were stained with Safranin-O to analyze the width of the growth plate by Osteomeasure system (Osteometrix).

Statistics

For statistical comparisons, the software package IBM SPSS statistics (version 22.0; IBM Corporation, Armonk, NY, United States) was used. Kolmogorov–Smirnov test using Lilliefors' correction was employed to test normal distribution of all acquired data sets. Outliers in normally distributed data sets were identified using Grubbs' test and excluded from further analysis (Grubbs, 1969). Outliers were identified in the following normally distributed datasets: absolute adrenal weight: Saline CSC (one animal), CSC recipient SHC (one animal); absolute thymus weight: saline CSC (one animal), CSC recipient SHC (one animal); distance moved NO: saline CSC (one animal). Normally distributed data sets were subsequently analyzed using parametric statistics, i.e., parametric Student's *t*-test [one factor, two independent samples; readouts: Physiology (absolute thymus weight; set 1)]; two-way ANOVA [two factors, two or more independent samples; readouts: Physiology (absolute adrenal weight, absolute thymus weight); Behavior (distance moved OF, time in corners OF, entries to OF, distance moved NO)]. Non-normally distributed data sets were analyzed using non-parametric statistics, i.e., Mann–Whitney *U* test [one factor, two independent samples; readouts: Physiology (body weight gain, plasma CORT, *in vitro* adrenal CORT production); Behavior (Time in corners NO, NO exploration); Intestinal inflammation (Histological damage score, F4/80 protein expression, CD11b protein expression, relative TNFα mRNA, relative IFNγ mRNA, relative CRAMP mRNA); Systemic inflammation (Plasma KC, plasma Il-6, plasma MCP-1); Bone homeostasis (Tibia length, growth plate thickness, trabecular thickness, trabecular BMD, bone vol./tissue vol., trabecular number)]; Kruskal–Wallis *H*-test [KWH-test; one factor, more than two independent samples; readouts: Physiology (body weight gain, plasma CORT); Behavior (Time in corners NO, NO exploration); Intestinal inflammation (Histological damage score, F4/80 protein expression, CD11b protein expression, relative TNFα mRNA, relative IFNγ mRNA, relative CRAMP mRNA); Systemic inflammation (Plasma KC, plasma Il-6, plasma MCP-1); Bone homeostasis (Tibia length, growth plate thickness, trabecular thickness, trabecular BMD, bone vol./tissue vol., trabecular number)]. All tests comparing more than two samples were followed, when a significant main effect was found, by *post hoc* analysis using Bonferroni pairwise comparison. For graphical illustration, the software package SigmaPlot (version 13.0; Systat Software Inc., San Jose, CA, United States) was used. Normally distributed data are presented as bars (mean + SEM). Non-normally distributed data are presented as box plots. Solid line represents the median, dashed line represents the mean for each data set. Lower box indicates 25th, upper box indicates 75th percentile, 10th (lower error bar) and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. The level of significance was set at $P < 0.05$.

RESULTS

Infusion of SHC Donor Feces Slightly Ameliorates CSC-Induced Behavioral Effects

Chronic subordinate colony housing increased anxiety-related behavior in the OF/NO test performed on day 19 of CSC (**Figures 1A–F**) in all groups compared to the respective SHC group, indicated by a significant main effect of the factor stress in the time spent in the corners during the OF test (two-way ANOVA; main effect for stress: $F_{1,76} = 18.207$; $P < 0.001$; Bonferroni: saline-infused: $P = 0.03$; SHC-infused: $P = 0.021$; CSC-infused: $P = 0.006$; **Figure 1B**) as well as the distance moved during the NO test (two-way ANOVA; main effect for stress: $F_{1,75} = 28.876$; $P < 0.001$; Bonferroni: saline-infused: $P = 0.016$; SHC-infused: $P = 0.018$; CSC-infused: $P < 0.001$; **Figure 1D**). Moreover, all CSC vs. SHC groups spent more time in the corners of the arena during the NO-test [Saline-treated: $P = 0.001$ (MWU); SHC-recipient: $P = 0.039$ (MWU); CSC-recipient: $P = 0.001$ (MWU); **Figure 1E**].

The number of entries to the open field (two-way ANOVA; main effect of stress: $F_{1,76} = 11.169$; $P = 0.001$; **Figure 1C**)

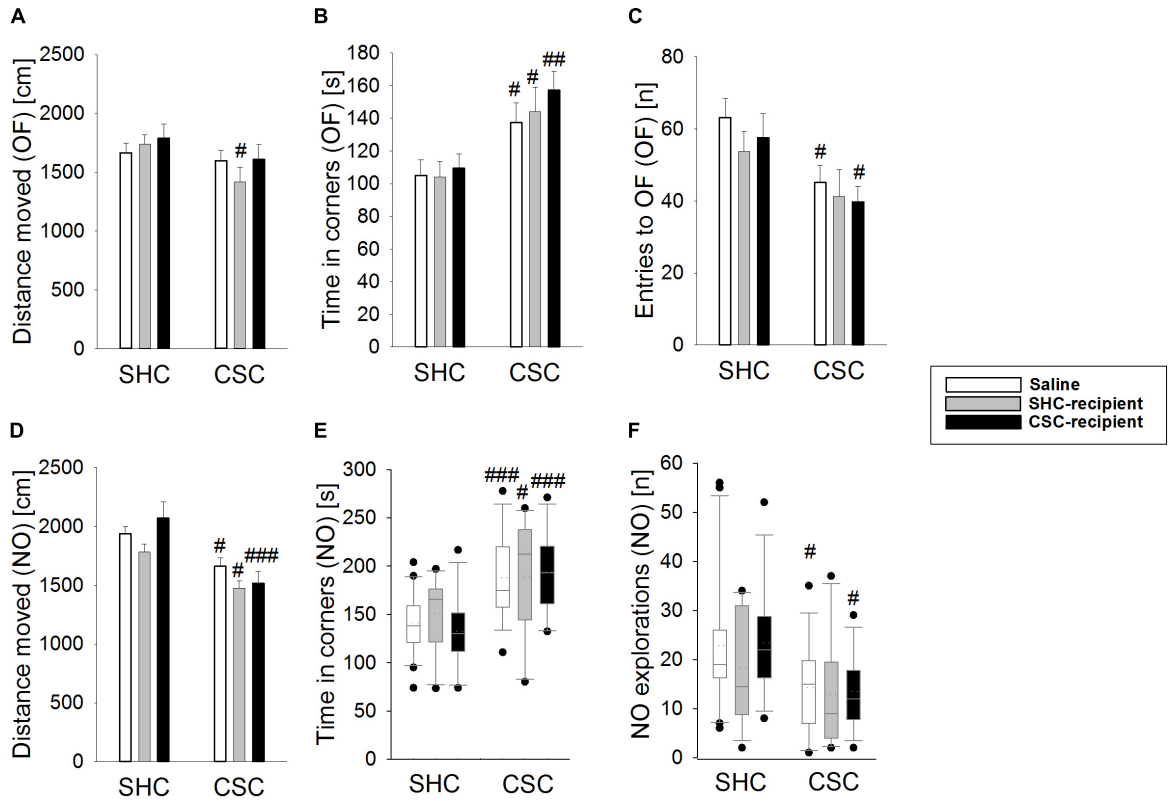

FIGURE 1 | Effects of fecal transplantation (FT) on chronic subordinate colony (CSC) housing-induced anxiety-related behavior. Anxiety-related behavior was assessed in the Open Field/Novel Object (OF/NO) test in the morning of day 19. Single-housed control (SHC)-infused CSC compared with respective SHC animals showed a decreased distance moved during the OF test **(A)**. Compared to the respective SHC groups, all CSC groups spent significantly more time in the corners during OF exposure **(B)**. In contrast to saline- and CSC-infused CSC mice, SHC-infused CSC did not show a decreased number of entries into the OF **(C)**. Compared to the respective SHC groups, all CSC groups showed a decreased locomotion **(D)** and an increased time in the corners during NO exposure **(E)** in the NO test. However, in contrast to saline- and CSC-infused CSC mice, SHC-infused CSC animals did not show a decreased number of NO explorations **(F)** during the NO test, when compared to their respective SHC group. SHC (n = 12–20); CSC (n = 12–14). Parametric data are presented as mean + SEM. Non-parametric data are represented as box-plots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile, 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ versus respective SHC.

was only significantly decreased in saline-treated CSC vs. SHC (Bonferroni: $P = 0.018$) as well as CSC-recipient CSC vs. SHC animals (Bonferroni: $P = 0.045$). Moreover, saline-infused CSC (MWU-test: $P = 0.027$) and CSC-recipient CSC (MWU-test: $P = 0.012$) but not SHC-recipient CSC animals displayed a reduced number of novel object explorations during the NO test (**Figure 1F**) when compared to their respective control group. However, SHC-recipient CSC vs. SHC mice also showed a shorter distance moved during the OF test (two-way ANOVA; main effect of stress: $F_{1,76} = 5.011$; $P = 0.028$; Bonferroni: $P = 0.04$; **Figure 1A**).

Infusion of SHC Donor Feces Slightly Ameliorates CSC-Induced Physiological Effects

Chronic subordinate colony housing increased the absolute adrenal weight in all groups compared to the respective SHC group (two-way ANOVA; main effect for stress: $F_{1,69} = 56.139$; $P < 0.001$; Bonferroni: saline-infused: $P < 0.001$; SHC-infused: $P < 0.001$; CSC-infused: $P = 0.003$; **Figure 2A**), indicating that CSC-induced changes in adrenal weight were independent of the microbiome. In contrast, while CSC-infused CSC (Bonferroni: $P = 0.05$) and saline-infused CSC (by trend: independent T-test: $P = 0.082$) mice displayed thymus atrophy when compared to respective SHC mice, SHC-infused CSC mice did not (two-way ANOVA; main effect for stress: $F_{1,73} = 6.283$; $P = 0.014$; **Figure 2B**). Body weight gain during CSC was neither affected by the factor stress, nor factor FT in any of the experimental groups (**Figure 2C**). CSC further increased plasma morning CORT in all CSC groups (MWU-test; saline-infused: $P < 0.001$, SHC-infused: $P = 0.033$; CSC-infused: $P = 0.014$) compared to respective SHC groups (**Figure 2D**), with this effect being less pronounced in SHC-infused CSC mice [KWH-test; H(2): 10.158; $P = 0.006$] compared with saline- (Bonferroni: $P = 0.022$) and CSC-infused CSC mice (Bonferroni: $P = 0.013$). Finally, all SHC (saline-infused: $P < 0.001$; SHC-recipient: $P < 0.001$,

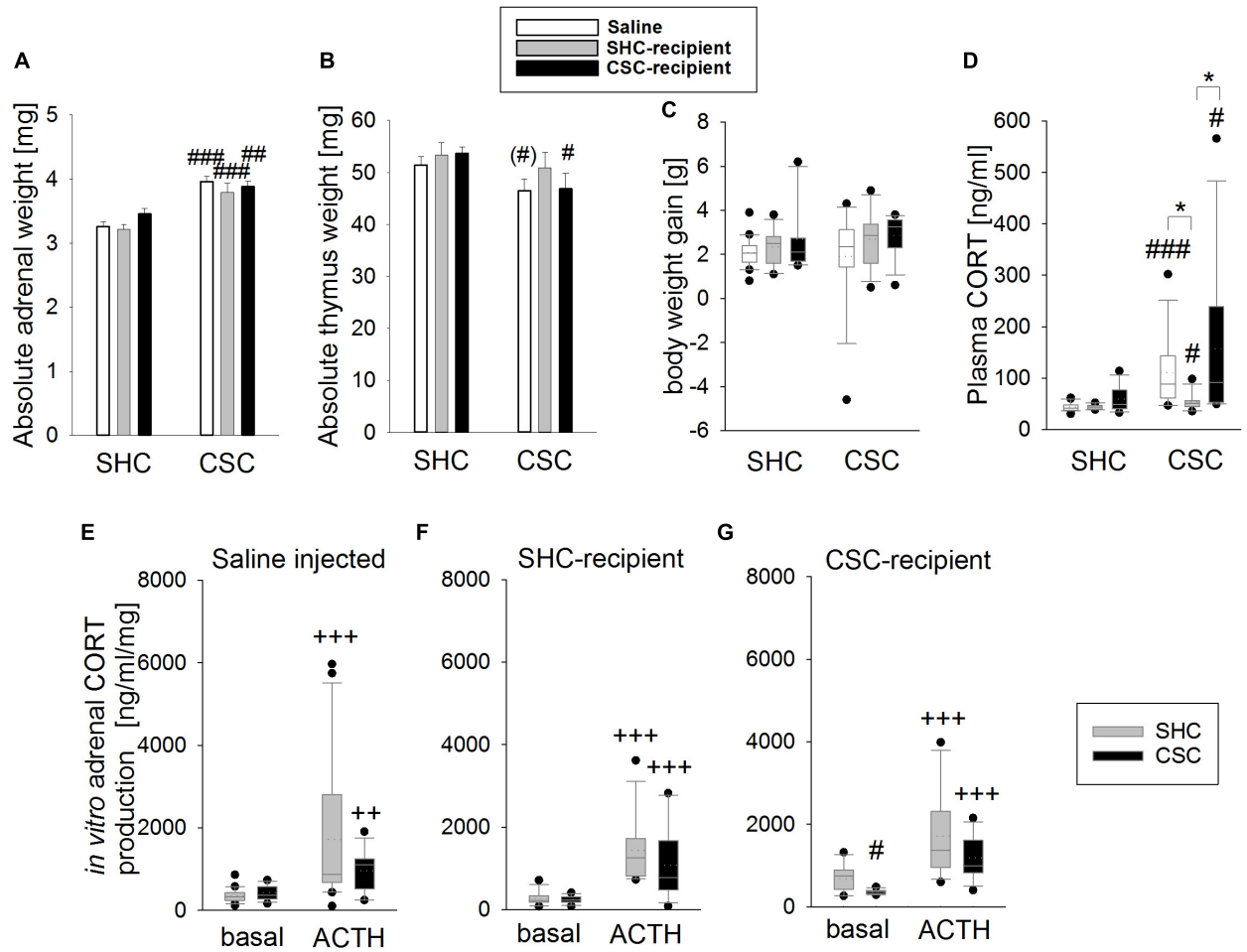

FIGURE 2 | Effects of fecal transplantation (FT) on chronic subordinate colony (CSC) housing-induced physiological alterations. Independent of FT, CSC resulted in an increased absolute adrenal weight **(A)** in all CSC vs. single-housed control (SHC) groups, while infusion of SHC donor feces was able to prevent CSC-induced thymus atrophy **(B)**. Body weight gain during CSC **(C)** was not affected by CSC and/or FT. Basal plasma corticosterone (CORT; **D**) levels were increased in all CSC vs. SHC groups, although this effect was less pronounced in SHC-recipient CSC vs. SHC mice. Moreover, all groups of CSC and SHC mice secreted comparable CORT concentrations in response to *in vitro* adrenocorticotropic hormone (ACTH; 100 nM) stimulation **(E–G)**. SHC (n = 11–20); CSC (n = 10–13). Parametric data are presented as mean + SEM. Non-parametric data are represented as box-plots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile, 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. $^{*}P \leq 0.05$ versus, respectively, indicated group; $^{(\#)}P$ = strong trend, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ versus respective SHC; $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ versus respective basal.

CSC-recipient: $P = 0.001$) and CSC (saline-infused: $P = 0.008$; SHC-recipient: $P < 0.001$, CSC-recipient: $P < 0.001$) groups produced higher amounts of CORT following ACTH stimulation (**Figures 2E–G**), when compared to their respective basal values. CSC-infused CSC vs. respective SHC mice produced lower amounts of CORT under basal conditions (MWU: $P = 0.013$).

CSC and FT Did Not Affect the Local Inflammatory Status of the Colon

Statistical analysis revealed no effect of the factors stress and/or FT on the histological damage score (**Figure 3A**), the colonic protein levels of F4/80 (**Figure 3B**) and CD11b (**Figure 3C**) as well as the mRNA levels of TNFα (**Figure 3D**), IFNγ (**Figure 3E**), and CRAMP (**Figure 3F**) in colonic tissue.

Infusion of SHC and CSC Donor Feces Affected CSC-Induced Systemic Inflammation in the Plasma, Respectively

Statistical analysis revealed that KC, IL-6 as well as MCP-1 levels were increased in the plasma of saline-infused CSC [MWU test: KC: $P = 0.016$ (**Figure 4A**); IL-6: $P = 0.004$ (**Figure 4B**); MCP-1: $P = 0.028$ (**Figure 4C**)] and CSC-recipient CSC (MWU test: KC: $P = 0.008$; IL-6: $P = 0.048$; MCP-1: $P = 0.004$) vs. respective SHC animals. SHC-recipient CSC animals only showed increased MCP-1 (MWU: $P = 0.029$) but not KC and IL-6 levels when compared to their respective controls. Statistical analysis using

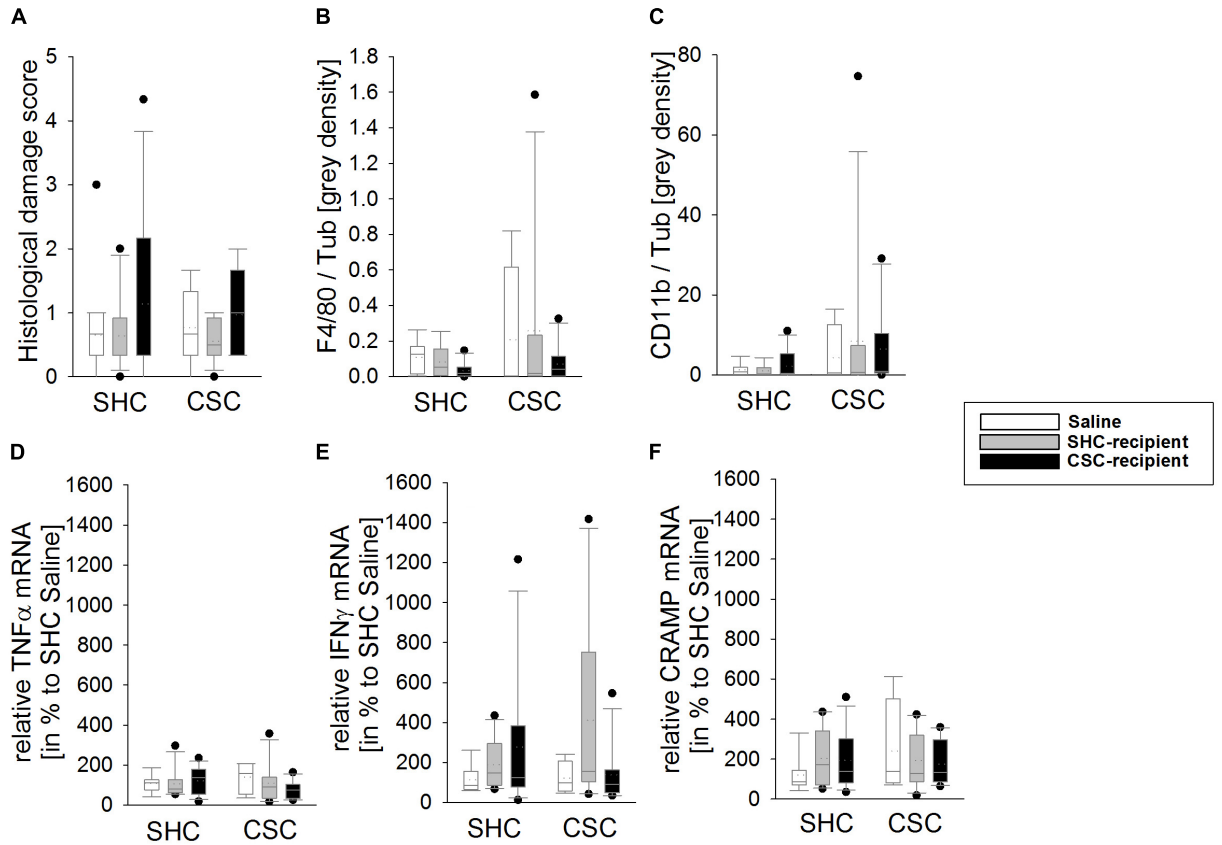

FIGURE 3 | Effects of fecal transplantation (FT) on local colonic inflammatory parameters. The inflammatory status locally in the colon of single-housed control (SHC) and chronic subordinate colony (CSC) housing mice was assessed at the histological, protein and mRNA level. There was no effect of CSC and/or FT on the histological damage score **(A)**, the colonic protein levels of F4/80 **(B)** and cluster of differentiation molecule 11b (CD11b; **C**) as well as the mRNA levels of tumor necrosis factor-α (TNFα; **D**), interferon-γ (IFNγ; **E**) and cathelin-related antimicrobial peptide (CRAMP; **F**) in colonic tissue. SHC (n = 7–19); CSC (n = 4–13). Parametric data are presented as mean + SEM. Non-parametric data are represented as box-plots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile, 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown.

KWH test further revealed a significant main effect of FT on plasma KC [H(2): 7.411; P = 0.025]; as well as plasma IL-6 levels [H(2): 7.449; P = 0.024] in CSC animals. Thereby, saline-infused CSC (Bonferroni: P = 0.055) as well as CSC-recipient CSC animals (Bonferroni: P = 0.055) had higher plasma KC levels than SHC-recipient CSC animals. Moreover, CSC-recipient CSC animals had significantly higher IL-6 levels than saline-infused CSC animals (Bonferroni: P = 0.042) and, by trend, higher IL-6 levels than SHC-recipient CSC animals (Bonferroni: P = 0.072).

Infusion of SHC Donor Feces Ameliorated CSC-Induced Effects on Bone Homeostasis. In Turn, Infusion of CSC Donor Feces Induced Stress Effects on Bone Homeostasis in Unstressed SHC Mice

Saline-infused CSC compared to SHC animals had significantly smaller tibia length (MWU: P = 0.024; **Figure 5A**) and an increased femoral growth-plate width (MWU: P = 0.038; **Figure 5B**). This effect was not visible in SHC-recipient and CSC-recipient CSC compared to SHC animals, respectively. Moreover, CSC-recipient SHC animals displayed significantly smaller tibia length compared to saline-infused SHC animals [KWH-test (H(2): 9.882; P = 0.007; Bonferroni: P = 0.005]. Statistical analysis further revealed, that trabecular thickness (**Figure 5C**), trabecular BMD (**Figure 5D**) as well as trabecular bone volume/tissue volume (BV/TV, **Figure 5E**) were significantly increased in both CSC vs. SHC saline-infused (MWU: Trabecular thickness: P = 0.048; BMD: P = 0.004; BV/TV: P = 0.008) as well as CSC-recipient animals (MWU: Trabecular thickness: P = 0.014; BMD: P < 0.001; BV/TV: P = 0.054). However, these effects were absent in SHC-recipient CSC vs. SHC animals. SHC-recipient CSC animals moreover displayed a significantly smaller trabecular thickness compared to saline-infused CSC animals [KWH-test: H(2): 9.723; P = 0.008; Bonferroni: P = 0.006] and a smaller trabecular BMD compared to CSC-recipient CSC animals [KWH-test: H(2): 8.406; P = 0.015; Bonferroni: P = 0.023]. Furthermore, CSC-recipient vs. saline-infused SHC animals displayed an increased trabecular BMD [KWH-test:

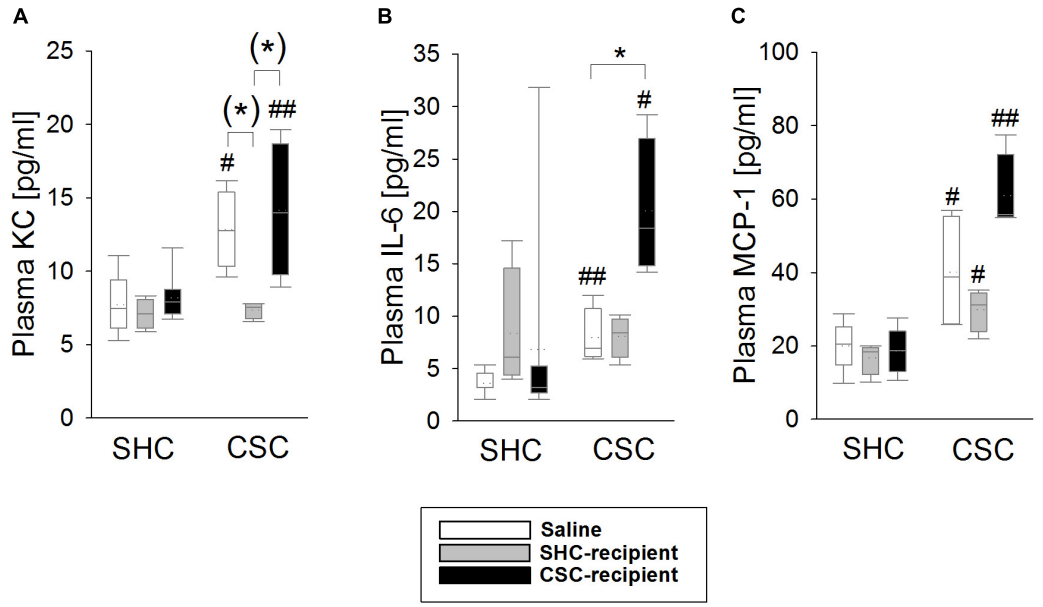

FIGURE 4 | Effects of fecal transplantation (FT) on chronic subordinate colony (CSC) housing-induced systemic inflammation. Systemic inflammatory markers were measured in the plasma of single-housed control (SHC) and CSC mice. Plasma levels of keratinocyte chemoattractant (KC; **A**), interleukin-6 (IL-6; **B**) as well as monocyte chemotactic protein-1 (MCP-1; **C**) levels were increased in saline-infused CSC compared with respective SHC and CSC-infused CSC compared with respective SHC animals. In contrast, SHC-infused CSC compared with respective SHC animals only showed increased MCP-1, but not KC and IL-6 levels, and had lower KC levels than saline- and CSC-infused CSC mice. SHC (n = 4–8); CSC (n = 4). Parametric data are presented as mean + SEM. Non-parametric data are represented as box-plots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile, 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. $^{(*)}P$ = strong trend, $^{*}P \leq 0.05$ versus, respectively, indicated group; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ versus respective SHC.

H(2): 11.836; $P = 0.003$; Bonferroni: $P = 0.002$], trabecular BV/TV [KWH-test: H(2): 10.023; $P = 0.007$; Bonferroni: $P = 0.006$] and trabecular number [KWH-test: H(2): 10.533; $P = 0.005$; Bonferroni: $P = 0.004$; **Figure 5F**].

DISCUSSION

In the present study, we showed that repeated FT from SHC donors into CSC-recipient mice has mild stress protective effects, ameliorating CSC-induced thymus atrophy, anxiety-related behavior, systemic low-grade inflammation as well as alterations in bone homeostasis. In turn, FT from CSC donors induced a "stressed" bone phenotype in SHC-recipient mice and slightly aggravated CSC-induced systemic low-grade inflammation and changes in bone homeostasis in CSC-recipient mice. In contrast to previous CSC studies (Reber et al., 2007; Uschold-Schmidt et al., 2012), basal plasma CORT concentrations were increased compared to respective SHC mice, and ACTH-stimulated *in vitro* CORT production was comparable between CSC mice of all groups, suggesting that the procedure of rectal infusion *per se* interferes with CSC-induced HPA axis activity.

Numerous studies showed that the gut microbiome plays an important role in the intestinal barrier homeostasis (Backhed et al., 2004; Bercik et al., 2012), the maturation and functionality of the immune system (Round and Mazmanian, 2009; Round et al., 2010; Olszak et al., 2012), bone homeostasis (Sjogren et al., 2012; Li et al., 2016; Novince et al., 2017), acute stress responsiveness (Sudo et al., 2004), neurogenesis (Ogbonnaya et al., 2015) as well as in the regulation of the host's behavior and mood (Cryan and Dinan, 2012; Stilling et al., 2014). Consequently, it is not surprising, that the transplantation of stool is able to affect the recipient's physiology (Sudo et al., 2004), immunology (Tian et al., 2016; De Palma et al., 2017) and behavioral characteristics like exploratory (Bercik et al., 2011; Collins et al., 2013) or depressive-like behavior (Kelly et al., 2016). Given the potential of CSC to alter the overall composition of the intestinal microbiome (Reber et al., 2016b), and, amongst others, to affect anxiety-related behavior (Langgartner et al., 2015), the first aim of the present study was to investigate if the anxiogenic effects of CSC can be prevented and/or transmitted to non-stressed mice by the transplantation of SHC and CSC feces, respectively. To do so, we analyzed the animal's anxiety-related behavior in the OF/NO test on day 19. In line with previous publications (Langgartner et al., 2015, 2017), CSC increased the anxiety-related behavior in saline-treated animals, ensuring that the procedure of rectal infusion *per se* did not have an effect in this context. In detail, saline-treated CSC versus SHC animals displayed an increased time in the corners of the arena and a decreased number of entries into the inner zone during OF testing as well as a decreased locomotion and novel object exploration and an increased time in the corners during the subsequent 5 min of NO exploration. Importantly, the total distance moved during the OF test did not differ between

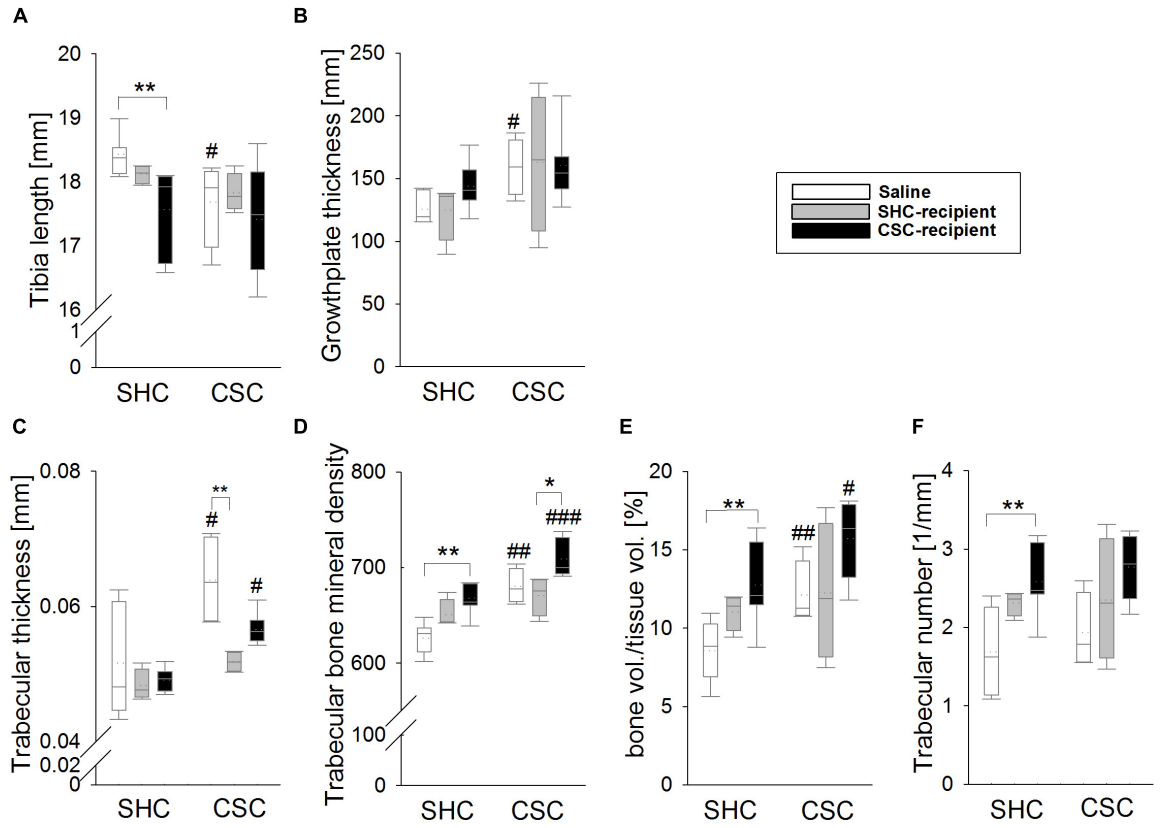

FIGURE 5 | Effects of fecal transplantation (FT) on chronic subordinate colony (CSC) housing-induced changes in the bone. Saline-infused CSC compared with respective single-housed control (SHC) mice had significantly shorter tibias **(A)** and an increased femoral growth-plate width **(B)**. Trabecular thickness **(C)**, trabecular bone mineral density (BMD) **(D)** as well as trabecular bone volume/tissue volume (BV/TV; **E**) were significantly increased in both saline-infused CSC compared with respective SHC mice as well as in CSC-infused CSC compared with respective SHC animals, but not in SHC-infused CSC versus SHC mice. Furthermore, the infusion of CSC feces compared with saline aggravated trabecular BMD, the trabecular BV/TV and the trabecular number **(F)** in SHC animals. SHC (n = 4–8); CSC (n = 4–8). Parametric data are presented as mean + SEM. Non-parametric data are represented as box-plots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile, 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ versus respectively indicated group; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ versus respective SHC.

saline-infused CSC and SHC animals, indicating that the outcome of the above mentioned anxiety-related parameters was not affected by differences in general locomotion (Langgartner et al., 2017). Interestingly, although infusion of CSC donor feces into both SHC and CSC recipients did not aggravate basal or CSC-induced anxiety-related behavior, infusion of SHC donor feces at least slightly ameliorated CSC-induced anxiety. More specific, infusion of SHC feces prevented the CSC-induced decrease in the numbers of entries into the inner zone and NO exploration during the OF and NO test, respectively. However, as SHC-infused CSC versus SHC mice displayed a decreased distance moved in the OF test, we at this point cannot exclude that the protective effects of SHC stool infusion on CSC-induced anxiety are affected by a general decrease in locomotion in these mice.

Adrenal hypertrophy and thymus atrophy are typical indicators of chronic stress in general (Selye, 1976; Engler and Stefanski, 2003; Stefanski et al., 2003; Ulrich-Lai et al., 2006), and of CSC in particular (Reber et al., 2007; Langgartner et al., 2015, 2017). In confirmation that the CSC model worked reliably in the present study, saline-infused CSC compared to SHC mice displayed an increased adrenal and decreased thymus weight. In line with the above reported anxiety data, infusion of CSC feces did not affect these parameters in SHC nor in CSC mice. However, in line with the hypothesis that infusion of SHC stool has stress-protective properties, SHC-infused CSC animals developed adrenal hypertrophy, but no thymus atrophy. In support of the latter, colonization of GF mice with conventional microbiota is able to influence the size of the thymus in a gender-specific manner (Fransen et al., 2017). Of note, body weight gain was neither affected by FT nor CSC, which is, at least for the latter, in line with previous studies indicating that a reduced body weight during CSC is not a very reliable parameter (Slattery et al., 2012; Langgartner et al., 2015).

Nineteen days of CSC have repeatedly shown to result in unaltered basal morning plasma CORT levels (Reber et al., 2007; Langgartner et al., 2017). All three groups of CSC mice in the current study, however, displayed increased basal CORT

concentrations compared to their respective SHC groups. Of note in this context, videotaping the CSC colony in the hour before euthanasia allows us to exclude that the elevated CORT levels in the present study are due to an acute attack by the resident shortly before killing. Given that not only SHC- and CSC-, but also saline-infused CSC mice showed this effect, we assume that the combination of rectal infusions and CSC exposure affected the HPA-axis in a different way than CSC exposure alone. In support, all groups of CSC mice did also not develop a decreased adrenal *in vitro* ACTH responsiveness (Uschold-Schmidt et al., 2012). Thus, although a recent study by Sudo et al. (2004) revealed that FT at an early developmental stage is able to alter the physiological stress-responsiveness toward acute stressors in mice, FT during adulthood seems to have little impact on HPA axis (re)activity, both during basal conditions and following chronic psychosocial stress.

Chronic psychosocial stress in humans is characterized by systemic immune activation and chronic low-grade inflammation (Bellingrath et al., 2013; Rohleder, 2014). Of note in this context, chronic psychosocial stress in male mice induced by the social disruption stress (SDR) paradigm, affects the gut microbial composition and, as a consequence, increased levels of circulating pro-inflammatory IL-6 and MCP-1 (Bailey et al., 2011). As 19 days of CSC also result in systemic low-grade immune activation, characterized by elevated concentrations of different pro- and anti-inflammatory mediators in the plasma (Langgartner et al., 2018) and changes in the gut microbial composition (Reber et al., 2016b), we hypothesized that these two CSC consequences are also causally linked with changes in the microbiome driving low-grade systemic inflammation. Confirming that the rectal infusion itself had no influence in this context, CSC reliably increased plasma levels of KC, IL-6 and MCP-1 in saline-infused mice. Importantly, and in support of the hypothesis that CSC-induced changes in the gut microbiome indeed are critically involved in promoting systemic inflammation, infusion with SHC feces prevented CSC-induced elevation of plasma KC and IL-6. Moreover, although in turn infusion of CSC donor feces did not induce a "stressed" cytokine profile in SHC mice, it aggravated the CSC-induced elevation of plasma IL-6.

In line with a recent study (Langgartner et al., 2017), CSC under SPF conditions in the present study did not cause any signs of local colonic inflammation, a finding that was not affected by the rectal infusion procedure *per se*. In detail, saline-infused CSC versus SHC mice did not differ in the histological damage score of the colon, the colonic mRNA expression of TNFα and IFNγ, as well as the colonic intestinal barrier function, indexed by colonic CRAMP mRNA expression. Moreover, colonic F4/80 and CD11b protein expression were also comparable between saline-infused CSC and SHC mice, suggesting no differences in the colonic macrophage and monocyte infiltration between the groups. In line with this lack of CSC-induced colitis, infusion of SHC or CSC mice with SHC or CSC feces did not result in any signs of colonic inflammation.

TABLE 1 | Summary of findings supporting the role of the intestinal microbiome as mediator of chronic psychosocial stress-induced pathology.

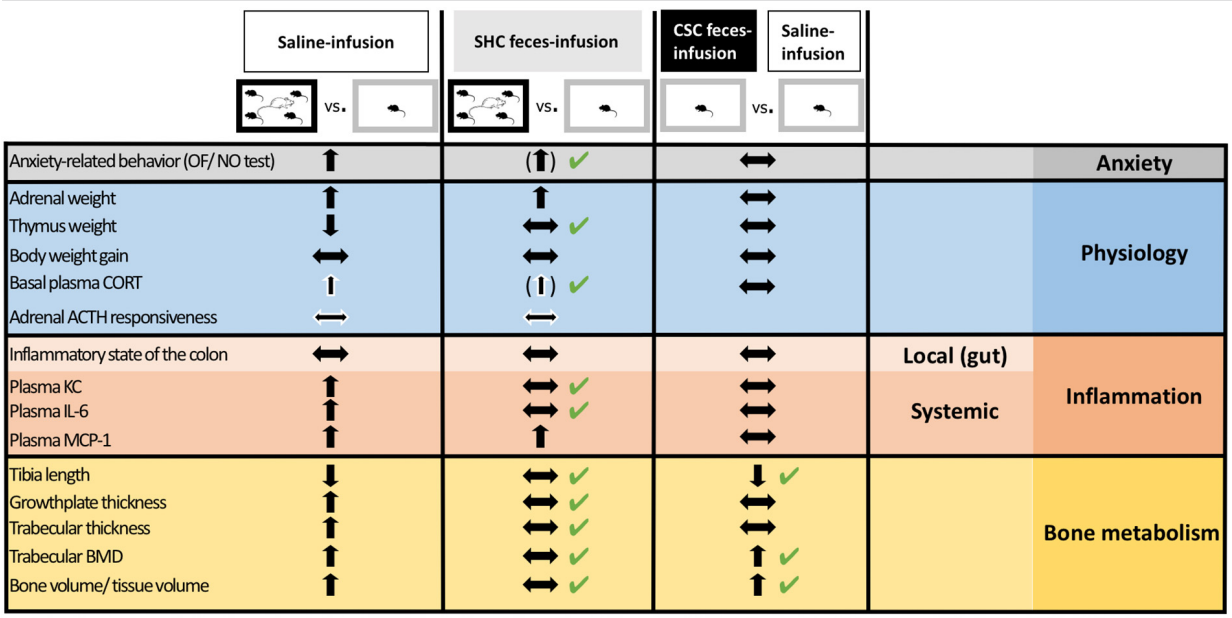

Table 1 *summarizes the main effects of single housed control (SHC)- and chronic subordinate colony housing (CSC)-feces-infusions on various behavioral, physiological, inflammatory and bone metabolic parameters in SHC and CSC mice. Black arrows indicate effect directions. White frames surrounding certain arrows in the left and middle columns indicate that CSC effects observed in "infused" mice in the present study are different from previously published CSC effects in "non-infused" mice. The left column compares saline-treated CSC animals with respective SHC mice. The middle column compares SHC feces-infused CSC with respective SHC mice. The right column compares CSC feces-infused SHC with saline-infused SHC mice. Green checkmarks in the middle and right column indicate findings supporting a causal role of the intestinal microbiome as mediator of CSC-induced pathology. Arrows in brackets represent partial but not full prevention/recovery.*

Besides being a risk factor for mental and inflammatory disorders, chronic stress or chronic stress-associated mental disorder as PTSD (Glaesmer et al., 2011, 2012; Calarge et al., 2014; Gebara et al., 2014; Zong et al., 2016) are linked to bone disorders (Batty et al., 2009; Azuma et al., 2015) and arthritis in humans. In line with this, our group was recently able to show that CSC negatively influences endochondral ossification (Foertsch et al., 2017), indicated by an increased appositional and decreased longitudinal bone growth. Confirming that the rectal infusion *per se* did not affect CSC effects on bone homeostasis, saline-infused CSC compared with respective SHC mice showed a decreased tibia length as well as an increased growth plate width, trabecular thickness, BMD and bone volume to tissue volume, similarly as described in our previous study (Foertsch et al., 2017). In support of the hypothesis that CSC-induced bone effects are mediated by the gut microbiome, infusion of SHC feces was able to prevent CSC effects on bone homeostasis. Moreover, infusion of CSC feces not only aggravated CSC-induced effects on trabecular BMD, it also induced a "stressed" bone phenotype in SHC-recipient mice. The latter was indicated by an increased BMD, BV/TV and trabecular number as well as a reduced tibia length in CSC-infused SHC mice compared with respective saline-infused SHC mice. Indeed, it was shown previously that the gut microbiome in general significantly effects on bone homeostasis and that alterations in the gut microbial community influence the latter. In detail, germ-free vs. conventionally raised mice exhibit increased bone mass, whereas re-colonization normalized bone parameters (Sjogren et al., 2012). Moreover, estrogen-depletion does not result in bone loss in germ-free mice and supplementation with probiotics prevents from ovariectomy-induced bone loss (Ohlsson et al., 2014; Li et al., 2016). Interestingly, changes in pro-inflammatory and pro-osteoclastic cytokines like TNFα, IL-6, and IL-1β were proposed as one underlying molecular mechanism (Ohlsson et al., 2014; Li et al., 2016; Xiao et al., 2017). Thus, the observed effects of FT on inflammatory cytokines in the present study might also, at least partly, account for the effects on bone homeostasis.

However, despite these promising results, there are still some limitations that have to be addressed in the future. In contrast to other human (Keshteli et al., 2017) and animal (Kelly et al., 2016) studies employing FT, we in the present study abstained from treating recipient animals with antibiotics prior to FT. Nevertheless, although antibiotic treatment decreases the overall gut microbial richness and diversity (Francino, 2015) and thus, potentially opens niches for infused germs to re-colonize the recipient's intestinal tract, it was recently shown in rodents, that the prior administration of antibiotics affects the establishment of transplanted donor phylotypes only in rather minor ways (Manichanh et al., 2010). However, it still remains unclear if this is also the case in our experimental setup. Another limitation is that we employed rectal instead of oral FT. The latter is more common in rodent studies (Bercik et al., 2011; Allen et al., 2012; Collins et al., 2013; Kelly et al., 2016; Galley et al., 2017) and also easily practicable in humans and thus of high translational relevance. Finally, it needs to be addressed if the FT method used in the present study is able to change the microbial composition of the host, and if so, what bacterial genera and species are involved in the protective/aggravating effects found in the present study.

CONCLUSION

In conclusion, although future studies are needed to optimize the stress-protective effects of FT, our findings revealed that infusion of "unstressed" donor feces might be a promising tool to prevent/treat the adverse outcomes of chronic psychosocial stress on systemic low-grade inflammation as well as bone homeostasis. Moreover, given that some negative consequences of chronic psychosocial stress could be transferred via FT, we propose that clinical approaches employing FT as a therapeutic tool, i.e., to treat recurrent *Clostridium difficile*-induced colitis (Dodin and Katz, 2014), should carefully screen fecal donor participants for their possible stress/trauma history. Thus, our findings (summarized in **Table 1**) on the one hand significantly contribute to understand the interplay between the gut microbiome and the host during chronic stress exposure and on the other hand expand the possible field of FT application.

AUTHOR CONTRIBUTIONS

DL and SR planned the study. DL, CV, A-LW, JK, SF, SB, and MH-L performed the experiments. DL and CV did the statistical analysis. DL, SF, MH-L, AI, and SR wrote the manuscript.

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Alterations of the Innate Immune System in Susceptibility and Resilience After Social Defeat Stress

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Dysregulation of innate immune responses has frequently been reported in stress-associated psychiatric disorders such as major depression. In mice, enhanced circulating cytokine levels as well as altered innate immune cell numbers have been found after stress exposure. In addition, stress-induced recruitment of peripheral monocytes to the brain has been shown to promote anxiety-like behavior. However, it is yet unclear whether specific differences in the innate immune system are associated with stress susceptibility or resilience in mice. Utilizing chronic social defeat, a model of depression and stress vulnerability, we characterized peripheral and brain-invading myeloid cells in stress-susceptible and resilient animals. In all defeated animals, we found reduced percentages of $CD11c^+$ dendritic cells (DCs) by flow cytometry in the spleen when compared to non-defeated controls. Exclusively in susceptible mice conventional DCs of the spleen showed up-regulated expression of MHC class II and co-stimulatory CD80 molecules pointing toward an enhanced maturation phenotype of these cells. Susceptible, but not resilient animals further exhibited an increase in inflammatory $Ly6C^{hi}$ monocytes and higher numbers of spleen-derived $CD11b^+$ cells that produced the proinflammatory cytokine tumor necrosis factor (TNF) upon lipopolysaccharide (LPS) stimulation. Increased percentages of peripheral $CD45^{hi}$ $CD11b^+$ cells immigrated into the brain of defeated mice, regardless of resilience or susceptibility. However, cellular infiltrates in the brain of susceptible mice contained higher percentages of CC chemokine receptor 2 ($CCR2^+$) $Ly6C^{hi}$ monocytes representing an inflammatory phenotype. Thus, we defined specific stress-related immune signatures involving conventional DCs and inflammatory $Ly6C^{hi}$ monocytes in susceptible and resilient mice. Together, our findings suggest an impact of the innate immune system in vulnerability to stress-related disorders such as major depression.

Keywords: chronic stress, social defeat, susceptibility, resilience, myeloid cells, major depressive disorder (MDD), monocytes, dendritic cells

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INTRODUCTION

Stressful experiences represent a major risk factor for mental illness such as major depressive disorder (MDD; Kessler et al., 2005; Krishnan and Nestler, 2008). However, physiological and psychological responses to stress vary and some individuals exhibit stress resilience (Krishnan and Nestler, 2008). Unraveling the underlying mechanisms of susceptibility and resilience is of major importance, due to the substantial burden that is associated with stress-associated MDD. In MDD patients, elevated levels of inflammatory markers including the proinflammatory cytokines tumor necrosis factor (TNF), interleukin (IL)-6, and IL-12 have been found in meta-analyses (Dowlati et al., 2010; Kohler et al., 2017). In addition to elevated cytokine levels, depressed patients show increased counts of neutrophils and monocytes that represent a prominent cellular source of these cytokines (Maes et al., 1992). However, it is an open question whether these immune changes are associated with stress vulnerability.

An established model to study stress susceptibility and resilience in mice is chronic social defeat. In this model, social avoidance behavior is induced in about one half of the animals while the others show social interaction comparable to controls (Krishnan et al., 2007). Susceptibility to social defeat stress has been shown to be mediated for instance by increased brain-derived neurotrophic factor (BDNF) signaling in mesolimbic dopamine (DA) pathways, enhanced firing rates of DA neurons, and activation of cortical projections from the prelimbic cortex to the nucleus accumbens (Berton et al., 2006; Krishnan et al., 2007; Cao et al., 2010; Vialou et al., 2014). Up to now, there is a lack of information on specific immune patterns especially involving myeloid cells associated with susceptibility and resilience in the chronic social defeat model. Only one study demonstrated that increased levels of IL-6 in mice predict and causally contribute to stress susceptibility (Hodes et al., 2014). However, innate immune changes specifically associated with stress-induced behaviors or resilience in this model are yet unresolved.

Other stress models in rodents revealed elevated levels of proinflammatory cytokines including TNF and IL-6, as well as the T helper cell differentiation cytokine IL-12 (Wohleb et al., 2011; Voorhees et al., 2013; Cheng et al., 2015). Acute and repeated stressors also increase neutrophil numbers (Engler et al., 2004a,b; Heidt et al., 2014; Lafuse et al., 2017). In addition, bone marrow-derived inflammatory $CD11b^{+}$ $Ly6C^{hi}$ monocytes increase in numbers after stress exposure (Wohleb et al., 2013; Heidt et al., 2014; Zheng et al., 2016; Lafuse et al., 2017). These cells have further been shown to immigrate into the brain of mice in response to repeated social disruption or foot shocks (Wohleb et al., 2011; Ataka et al., 2013). While these data point toward an important role of the innate immune response in stress and stress-associated behaviors, specific features of myeloid cells associated with stress vulnerability and resilience in the chronic social defeat model have not been studied.

The aim of this study was to identify specific innate immune cell profiles induced by chronic social defeat and to elucidate whether they are associated with stress vulnerability. We conducted flow cytometric analyses on *ex vivo* isolated spleen- and CNS-derived myeloid cells and intracellular cytokine staining after *in vitro* restimulation on peripheral myeloid cells of susceptible and resilient mice. Here we report specific innate immune signatures associated with stress vulnerability in these mice.

MATERIALS AND METHODS

Mice and Housing Conditions

Male C57BL/6J mice were purchased at Charles River (Sulzfeld, Germany) at the age of 5 weeks and directly introduced into the experimental room. Animals were housed in groups of four in Makrolon type II-L cages (365 × 207 × 140 mm) for a period of 2 weeks before the experiments started. Male CD-1 mice of at least 3 months of age were used as resident animals for the social defeat paradigm and as partners in the social interaction test. Most of these mice had mating experience prior to the inclusion into this study. In addition, resident mice were checked for their aggressive behavior (latency to attack intruder should be less than 30 s). CD-1 males were housed singly in Makrolon type III cages (425 × 266 × 155 mm) until the experiment started. The experimental room was maintained at a temperature of 22 ± 2°C, humidity of 55 ± 10% and a 12 h:12 h light-dark cycle, with lights on at 6 am. Food and water were available *ad libitum*. This study was repeatedly performed in three independent cohorts with the addition of some additional parameters after the first cohort. Supplementary Table S1 gives an overview about sample sizes and analyzed parameters for each cohort. Within any cohort, control and social defeat mice were investigated and dissected at the same time. This study was performed in accordance with the regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 2010/63/EU). The project was approved by the local authority (LANUV NRW) and the Animal Welfare Officer of the University of Münster. All efforts were made to minimize animal suffering and reduce the number of animals used.

Social Defeat Paradigm

The social defeat paradigm applied in this study was based on the paradigm by Berton et al. (2006) with slight modifications in the setup of the social confrontation cage. All experimental mice were inserted into the cage of an aggressive, older and heavier CD-1 mouse for 10 min per day. After 10 min of direct physical contact, animals were separated by a perforated Plexiglas wall and kept on opposite sides of the same cage for 24 h. Thus, visual and olfactory contact between the animals was maintained, while physical contact and the danger of injuries were avoided. This procedure was repeated daily, every day with a novel, unfamiliar dominant CD-1 opponent. After the final confrontation, experimental mice where housed singly in Makrolon type II cages (267 × 207 × 140 mm). All experiments comprised 10 confrontations on subsequent days. The confrontations were performed in Makrolon type III cages (425 × 266 × 155 mm) which were inhabited by a CD-1 mouse for at least three days such that the cage could be considered its territory. The cages were modified in a way that a perforated Plexiglas wall could be introduced to separate the

cage in two parts of equal size where on one side an additional water bottle and food pellets for the defeated mouse could be presented.

All defeat sessions have been observed by an experienced experimenter who carefully monitored the 10 min sessions and rated the degree of agonistic interactions on a scale between 0 and 3 based on the following definitions:

0. No agonistic encounters between the animals.
1. At least one agonistic encounter.
2. At least three bouts of the CD-1 mouse chasing the C57BL/6 intruder.
3. At least five bouts of chasing behavior or start of escalated fighting.

The experimenter terminated the sessions and separated the animals with the perforated Plexiglas divider as soon as escalated fighting occurred, even before 10 min passed. By this method, wounding of mice was successfully prevented to avoid potential influences of wounding on the innate immune response. However, few superficial scratches were equally found in susceptible and resilient animals. In a recent study, exclusively wounding, but not minor scratches, was associated with glucocorticoid resistance in splenocytes in a model of chronic social stress. Superficial scratches (considered non-wounded) did not induce further innate immune responses in addition to the stressor (Foertsch et al., 2017).

Control mice were housed in the same type of cage as experimental mice; however, two control mice were housed on the opposite sides of the perforated Plexiglas separation. To avoid effects induced by daily handling or cage change that was applied in experimental mice, control mice were daily handled and weighed. After weighing, control mice were put to the opposite compartments of the Plexiglas wall every day. After 10 days control mice were also housed singly.

Social Interaction Test

One day after the last social defeat session, the social interaction test was conducted as described before (Ambrée et al. (2016), referred to as social exploration test). Briefly, it comprised two trials of 150 s each. During the first trial the enclosure in the social exploration box was empty; in the second trial (social exploration trial) an unfamiliar CD-1 mouse (male, between 3 months and 5 months of age) was present inside the exploration enclosure in the box. The time spent in the interaction zone, defined as the area surrounding the exploration enclosure 8 cm to each side, was recorded in both trials by ANY-maze tracking software (Stoelting, Dublin, Ireland). An interaction ratio was calculated as time spent in the interaction zone during the social exploration trial divided by the time spent in the zone during the first trial. Based on the interaction ratio, animals were defined as susceptible when the ratio was less than 0.5. In case the interaction ratio was greater than 0.5, animals were defined as resilient. The threshold of 0.5 was chosen to define resilient animals because the interaction ratio of control mice was in the same range with a mean around 1 and the smallest values starting around 0.5.

Flow Cytometry of Splenocytes

After transcardial perfusion with ice-cold PBS, spleens were homogenized by digestion with collagenase type VIII and DNase I (Sigma-Aldrich, Munich, Germany) for 45 min at 37°C before preparation of a single-cell suspension. Cells were counted using a CASY cell counter (Roche, Mannheim, Germany). Following collection, cells were stored on ice for further use. Fluorescence staining was performed using the following antibodies purchased from Biolegend or BD Biosciences: PE-conjugated anti-mouse Ly6G (clone RB6-8C5), PerCP-Cy5.5-conjugated anti-mouse CD80 (clone 16-10A1), PE-Cy7-conjugated anti-mouse Ly6C (clone HK1.4), APC-conjugated anti-mouse MHC-II (clone M5/114.15.2), eFluor 450-conjugated anti-mouse CD11c (clone N418), BV510-conjugated anti-mouse CD11b (clone M1/70). Fc receptors were blocked with antibodies against mCD16/CD32 (Biolegend, San Diego, CA, USA). Samples were acquired on a FACSCanto II (BD Biosciences, East Rutherford, NJ, USA) flow cytometer and analyzed using FlowJo v10. Electronic gating included life gates to exclude debris and dead cells and additional gating strategies to discriminate doublets from single cells. The gating strategies to determine myeloid cell subsets are described in **Figures 2A**, **3A** and Supplementary Figure S2.

In Vitro Stimulation of Splenocytes With LPS

Splenocytes (six wells/animal containing 5×10^5 cells/100 μl) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FCS, L-glutamine, penicillin/streptomycin, 2-mercaptoethanol (all from Gibco) and stimulated for 10 h with 100 ng/ml *Escherichia coli* Lipopolysaccharide (LPS) Serotype O127:B8 (Sigma Aldrich, St. Louis, MO, USA) plus Monensin and Brefeldin A (Biolegend, San Diego, CA, USA). Fluorescence staining was performed using the following antibodies purchased from Biolegend or BD Biosciences: PE-conjugated anti-mouse IL-12 p40/p70 (clone C15.6), biotinylated anti-mouse CD11b (clone M1/70) and Streptavidin conjugated to PerCP/Cy5.5, APC-conjugated anti-mouse TNF α (clone MP6-XT22), eFluor 450-conjugated anti-mouse CD11c (clone N418). Fc receptors were blocked with antibodies against mCD16/CD32 (Biolegend, San Diego, CA, USA). Intracellular staining was performed according to the manufacturer's instruction using the Fixation/Permeabilization kit (BD Cytofix/Cytoperm). Samples were acquired on a FACSCanto II (BD Biosciences, East Rutherford, NJ, USA) flow cytometer and analyzed using FlowJo v10 (TreeStar™). Electronic gating included life gates to exclude debris and dead cells and additional gating strategies to discriminate doublets from single cells. The gating strategies to determine cytokine producing myeloid cell subsets are described in **Figure 4A** and Supplementary Figure S2.

Flow Cytometry of CNS Mononuclear Cells

Mice were perfused with ice-cold PBS to remove leucocytes from intracerebral blood vessels. Brains were homogenized by manual disruption and incubation in Collagenase/Dispase, and DNase I at 37°C for 45 min each (Roche Applied Science, Mannheim, Germany). Mononuclear CNS cells were enriched by gradient centrifugation of 30% and 70% Percoll (GE Healthcare, Ltd.,

Buckinghamshire, UK). Enriched cells were collected from the 30%/70% interface of the Percoll gradient after centrifugation at 921× *g* for 25 min at room temperature. Cells were counted using a CASY cell counter (Roche, Mannheim, Germany). Following collection, cells were stored on ice for further use. Fluorescence staining was performed using the following antibodies purchased from Biolegend or BD Biosciences: PE-conjugated anti-mouse CCR-2 (clone 475301), PE-Cy7-conjugated anti-mouse Ly6C (clone HK1.4), APC-Cy7-conjugated anti-mouse CD45 (clone 30-F11), eFluor 450-conjugated anti-mouse CD11c (clone N418), BV510-conjugated anti-mouse CD11b (clone M1/70). Fc receptors were blocked with antibodies against mCD16/CD32 (Biolegend, San Diego, CA, USA). Samples were acquired on a FACSCanto II (BD Biosciences, East Rutherford, NJ, USA) flow cytometer and analyzed using FlowJo v10 (TreeStar^{TM}). Electronic gating included life gates to exclude debris and dead cells and additional gating strategies to discriminate doublets from single cells. The gating strategies to determine intracerebral leucocyte subsets are described in **Figures 5A,B** and Supplementary Figure S2.

Statistics

For the statistical analysis, data obtained in three independent cohorts were combined and analyzed by analysis of covariance (ANCOVA) with stress phenotype as fixed factor and cohort as covariate. In case of significant effects of the stress phenotype, Bonferroni posthoc tests were calculated. To support findings from the ANCOVA analysis, Pearson correlations were calculated between immune parameters that were differentially affected in susceptible and resilient mice and measures of social avoidance. Immune parameters were transformed to percent of the control mean within each cohort to correct for inter cohort variability. The null-hypothesis was rejected for $p < 0.05$. All analyzes were calculated with SPSS 24 (IBM).

RESULTS

Social Defeat Stress Is Associated With Social Avoidance in Susceptible Mice

To investigate the effects of stress on the innate immune response, we subjected 7 week old C57BL/6 mice to 10 days of social defeat stress (**Figure 1A**). One day later, the exploration of an unfamiliar social partner was assessed in the social interaction test. Based on the definition of susceptible and resilient mice, social defeat stress resulted in a markedly reduced interaction ratio in susceptible mice compared to controls ($p < 0.001$) and resilient mice ($p < 0.001$, **Figure 1B**). The reduced exploration of the conspecific was associated with increased time spent in the opposite corners of the apparatus (**Figures 1C–E**), with susceptible mice spending significantly less time in the interaction zone and more time in the corners as controls ($p < 0.001$) and resilient animals ($p < 0.001$).

With regard to physiological stress responses, body weight was similar between the groups at the beginning and the end of the experiment. At the time of tissue harvesting, basal corticosterone levels were increased exclusively in susceptible mice compared to resilient or control animals (C vs. S: $p < 0.01$; S vs. R: $p < 0.01$, Supplementary Figure S1). In contrast, the total number of thymocytes was significantly reduced in both defeated groups when compared to controls (C vs. S: $p < 0.05$; C vs. R: $p < 0.01$). These data indicate that a chronic stress response occurred in all defeated mice while corticosterone levels were only affected in susceptible mice.

To exclude whether the susceptible or resilient phenotype could be influenced by heterogeneity in fighting behavior during sessions, intensity of agonistic interactions was assessed by daily scoring. Susceptible and resilient mice showed equivalent numbers of agonistic encounters during defeat sessions indicating that behavioral outcome does not result from variations in aggressive behavior (Supplementary Figure S1E). These findings support the notion that the resilient phenotype does not result from fewer aversive experiences during the 10-day stress phase.

Altered Phenotype of Conventional Dendritic Cells in Stress Susceptible Mice

To analyze the effects of social defeat stress on the splenic immune cell composition and its potential association with stress susceptibility or resilience, we first quantified total numbers of mononuclear cells in the spleen. There was a significant difference in the absolute cell number per spleen between the groups (**Figure 2B**, $F_{(2,41)} = 4.47$, $p = 0.018$). In contrast to resilient mice, susceptible animals showed a significant increase in cellularity with higher total cell numbers in their spleens when compared to control mice ($p = 0.015$).

To study whether cells of the innate immune system are specifically affected by susceptibility or resilience to stress, we first focused on CD11c$^+$ dendritic cells (DCs). Both stress groups had markedly reduced percentages of CD11c$^+$ DCs in the spleen compared to controls (**Figure 2C**, C vs. S: $p < 0.001$; C vs. R: $p < 0.001$). The expression levels of cell surface molecules associated with antigen presentation and co-stimulation, MHC class II and CD80, were increased on DCs of susceptible mice compared to controls (**Figures 2D,E**, MHC class II: $p = 0.003$; CD80: $p = 0.017$).

We further quantified proportions of conventional CD11b$^+$ CD11c$^+$ DCs as well as CD11b$^-$ CD11c$^+$ DCs in this model. While percentages of CD11b$^-$ CD11c$^+$ DCs were equivalently increased in both stress groups (C vs. S: $p < 0.001$; C vs. R: $p < 0.001$), the subset of conventional CD11b$^+$ CD11c$^+$ DCs was reduced in these animals (**Figure 2F**, C vs. S: $p < 0.001$; C vs. R: $p < 0.001$). As shown for the total population of CD11c$^+$ DCs, surface expression levels of MHC class II and CD80 molecules were increased in conventional CD11b$^+$ CD11c$^+$ DCs of susceptible mice, but not in resilient and control animals (**Figure 2G**, MHC class II: $p = 0.008$; CD80: $p = 0.001$). With regard to the CD11b$^-$ CD11c$^+$ DC subset, these cells showed higher surface expression of MHC class II in the spleen of susceptible mice compared to control mice ($p = 0.001$). These data indicate that equally reduced proportions of conventional DCs occur in stress resilient as well as in susceptible mice. However, levels of MHC class II and

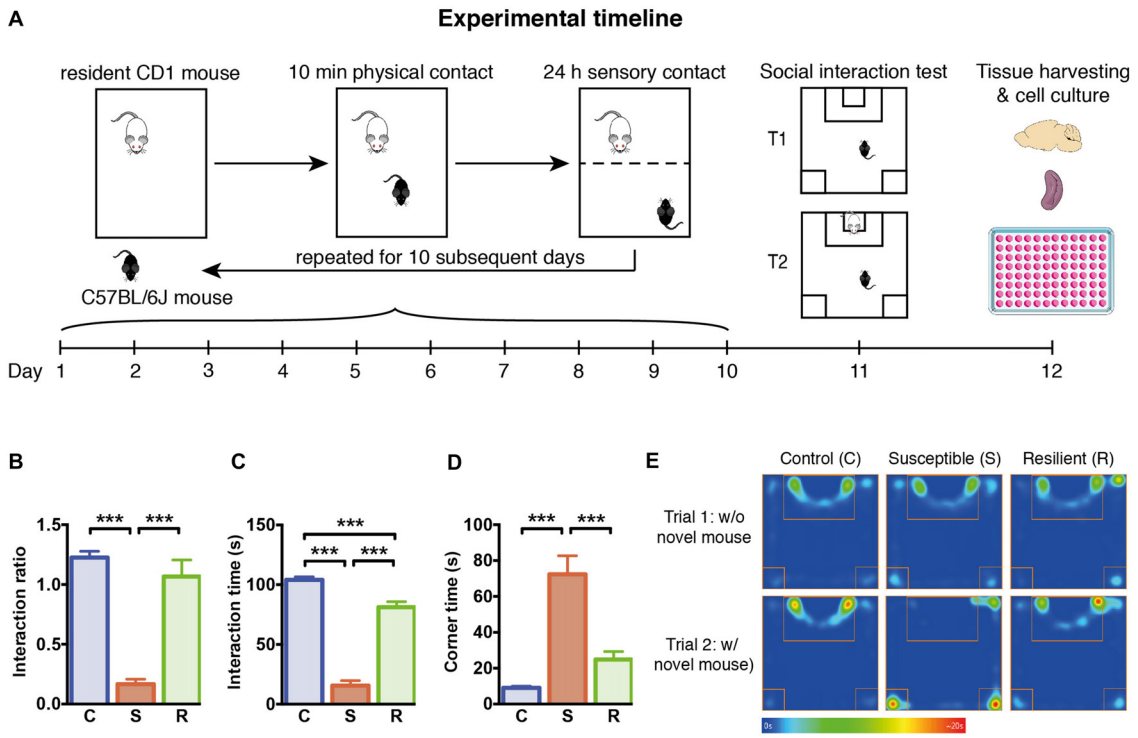

FIGURE 1 | Experimental timeline and social behavior. **(A)** Graphical representation of the experimental timeline. C57BL/6J mice were subjected to 10 min daily confrontation with CD-1 mice for 10 subsequent days. In between, mice were housed in the same cages separated by a perforated Plexiglas wall from the dominant and aggressive conspecific. At day 11, mice were tested in the social interaction test, before they were sacrificed 1 day later for tissue harvesting and cellular analysis. **(B)** Significantly reduced interaction ratio in susceptible mice calculated as time spent in the interaction zone in trial 2 in relation to the time in trial 1. **(C)** Significantly decreased time spent in the interaction zone during the 2nd trial of the social interaction test in susceptible mice. **(D)** Significantly increased time spent in the corners opposite to the interaction zone during the 2nd trial of the social interaction test in susceptible mice. **(E)** Mean heat map of the groups' center points for control, susceptible and resilient mice in the 1st and 2nd trial of the social interaction test. Bar graphs represent mean + SEM. T1, trial 1; T2, trial 2; C, control; S, susceptible; R, resilient. $n_C = 16$, $n_S = 15$, $n_R = 15$. $^{***}p < 0.001$.

co-stimulatory CD80 molecules are upregulated on conventional DCs exclusively in the spleen of susceptible mice.

Inflammatory Ly6C^{hi} Monocytes Prominently Increase in Stress Susceptible Mice

As mentioned before, spleen cellularity increased in susceptible mice compared to control animals. As a result absolute numbers of DCs were not altered ($F_{(2,26)} = 0.09$, $p = 0.913$, Supplementary Table S2). These data indicate that other non-DC cell populations expand in stress susceptibility. Accordingly, susceptible animals showed significantly elevated numbers of CD11b$^+$ myeloid cells that were negative for CD11c in the spleen compared to controls (**Figure 3B**, $p = 0.006$). A mild but non-significant increase in these cells was also detectable in resilient mice. About half of these cells could be identified as Ly6G^{hi} Ly6C^{low} neutrophils, which showed significantly higher numbers in susceptible and resilient mice (**Figure 3C**, C vs. S: $p = 0.004$; C vs. R: $p = 0.048$). The Ly6C^{hi} Ly6G^{low} subset of CD11b$^+$ CD11c$^-$ myeloid cells has been classified before as inflammatory monocytes (Gordon and Taylor, 2005). Susceptible mice presented elevated numbers of this cell type compared to controls (**Figure 3D**, $p = 0.019$). Together, these data indicate stress-induced alterations of the innate immune compartment in all defeated animals. However, a substantial increase in inflammatory Ly6C^{hi} monocytes specifically occurred in susceptible mice.

Enhanced Percentages of TNF-Producing CD11b$^+$ Cells in Stress Susceptible Mice

We explored the functional capacity of myeloid cells in stress susceptible and resilient mice and determined the capacity of these cells to produce IL-12 and TNF in response to Toll-like receptor ligand LPS. For this, we performed intracellular cytokine staining of splenic myeloid cells upon *in vitro* LPS stimulation and assessed the percentage of CD11c$^+$ DCs and CD11b$^+$ cells negative for CD11c that produced IL-12 and TNF. Resilient mice exhibited a higher percentage of DCs that produced IL-12 when compared to controls (**Figure 4B**, $p = 0.039$). In susceptible mice a mild but not significant increase was observed. The percentages of TNF-producing DCs were similar between all groups ($F_{(2,38)} = 0.21$, $p = 0.811$).

With regard to CD11b$^+$ myeloid cells, equivalent percentages of IL-12 producing cells were found in stressed animals and

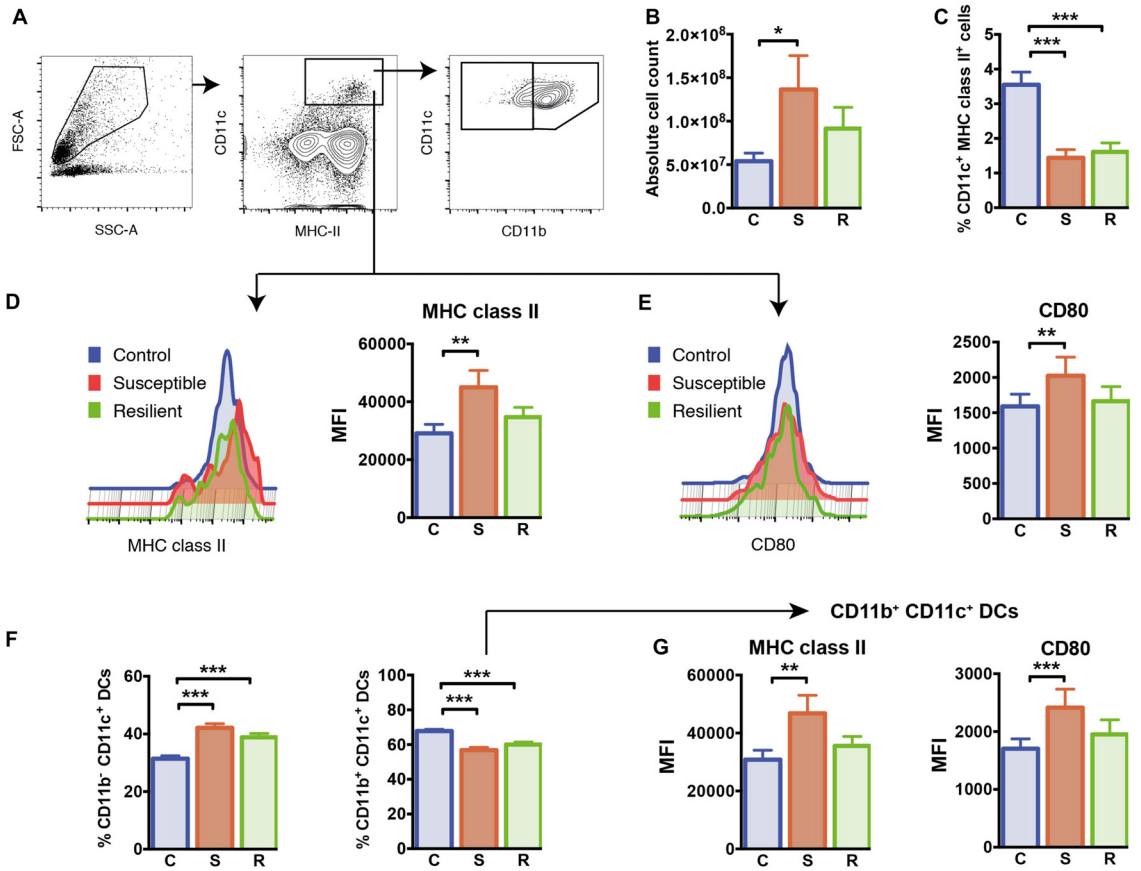

FIGURE 2 | Cellularity and dendritic cells (DCs) in the spleen. **(A)** Gating of CD11c$^+$ MHC class II$^+$ DCs and CD11b$^+$/CD11b$^-$ DC subsets. **(B)** The absolute cell numbers isolated from the spleens of experimental mice. **(C)** Reduced percentages of CD11c$^+$ MHC class II$^+$ DCs in mice subjected to social defeat. **(D)** Elevated expression of MHC class II molecules in susceptible mice pregated for CD11c$^+$ MHC class II$^+$ DCs. **(E)** Elevated expression of CD80 molecules in susceptible mice pregated for CD11c$^+$ MHC class II$^+$ DCs. **(F)** Increased percentages of the CD11b$^-$ CD11c$^+$ DC subset and decreased percentages of the CD11b$^+$ CD11c$^+$ DC subset in socially defeated mice. **(G)** Elevated expression of MHC class II molecules and CD80 molecules in CD11b$^+$ CD11c$^+$ DCs of susceptible mice. Bar graphs represent mean + SEM. C, control; S, susceptible; R, resilient; MFI, mean fluorescence intensity. Absolute cell count: $n_C = 16$, $n_S = 15$, $n_R = 15$, all other graphs in this Figure: $n_C = 10$, $n_S = 11$, $n_R = 9$. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

controls ($F_{(2,38)} = 0.40$, $p = 0.675$). However, in the spleen of susceptible mice higher percentages of TNF-producing CD11b$^+$ cells were found (**Figure 4C**, $p = 0.033$ vs. C). These data indicate that stress susceptibility is associated with increased percentages of CD11b$^+$ cells producing the inflammatory cytokine TNF, while resilience is associated with higher percentages of DCs producing the T cell differentiation cytokine IL-12.

Increased Brain Recruitment of Inflammatory CCR2$^+$ Ly6C^{hi} Monocytes in Susceptibility

Finally, we quantified microglia and brain infiltrating peripheral myeloid cells in the brain of these animals. Social defeat stress did not affect the percentage of CD45^{int} CD11b$^+$ microglia compared to controls (**Figure 5C**, $F_{(2,39)} = 1.17$, $p = 0.322$). However, percentages of CNS invading CD45^{hi} CD11b$^+$ myeloid cells that comprise peripheral monocytes/macrophages were significantly increased in susceptible mice (**Figure 5D**, $p = 0.001$). Resilient mice showed a mild increase in CD45^{hi} CD11b$^+$ cell percentages, that did not reach statistical significance ($p = 0.053$ vs. C). Among CD45^{hi} CNS infiltrates, DC percentages were significantly higher in defeated mice (**Figure 5E**, C vs. S: $p < 0.001$; C vs. R: $p = 0.004$). Percentages of infiltrating CC chemokine receptor 2 (CCR2$^+$) Ly6C^{hi} inflammatory monocytes were elevated in susceptible mice (**Figure 5F**, $p = 0.007$ vs. C) again hinting toward an activated immune status in susceptible defeated mice.

Innate Immune Parameters Correlate With Measures of Social Avoidance

To support the findings that specific parameters of the innate immune system are associated with stress susceptibility or resilience, we calculated correlations between the respective immune parameters and measurements of social avoidance. With the exception of percentages of splenic Ly6G^{hi} neutrophils

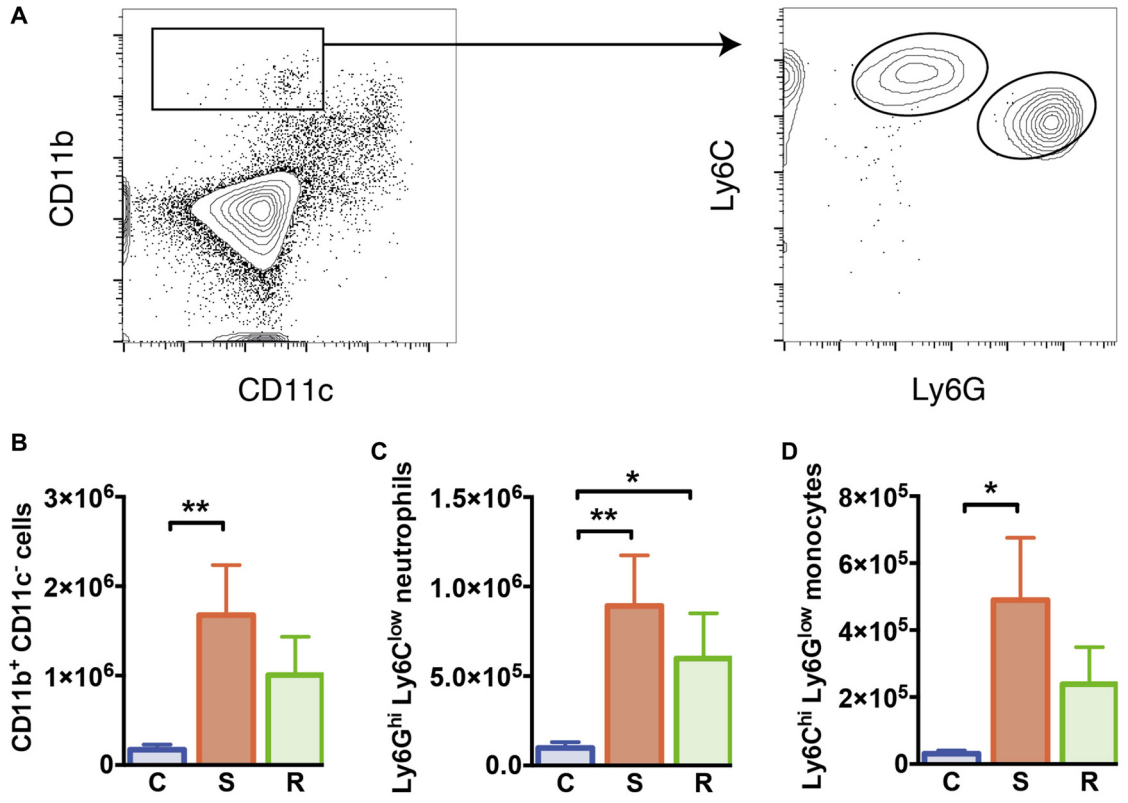

FIGURE 3 | Monocytes and neutrophils in the spleen. **(A)** Gating of CD11b$^+$ CD11c$^-$ myeloid cells, Ly6C^{hi} Ly6G^{low} inflammatory monocytes, and Ly6G^{hi} Ly6C^{low} neutrophils. **(B)** Increased numbers of CD11b$^+$ CD11c$^-$ myeloid cells in susceptible mice. **(C)** Increased numbers of Ly6G^{hi} Ly6C^{low} neutrophils in socially defeated mice. **(D)** Increased numbers of Ly6C^{hi} Ly6G^{low} inflammatory monocytes in susceptible mice. Bar graphs represent mean + SEM. C, control; S, susceptible; R, resilient. $n_C = 10$, $n_S = 11$, $n_R = 9$. $^*p < 0.05$, $^{**}p < 0.01$.

and stimulated IL-12-producing DCs, all other immune parameters differentially affected in susceptible or resilient mice showed significant correlations with the interaction ratio and the interaction time (Supplementary Table S3). Significant correlations include expression levels of MHC class II and CD80 on DCs, percentages and numbers of CD11b$^+$ monocytes as well as numbers of Ly6C^{hi} inflammatory monocytes. Furthermore, percentages and numbers of stimulated TNF producing CD11b$^+$ monocytes correlated significantly with social avoidance. This also holds true for the percentages of CD45^{hi} CD11b$^+$ brain invading peripheral myeloid cells and percentages of CCR2$^+$ Ly6C^{hi} CD45^{hi} brain invading inflammatory monocytes.

DISCUSSION

In this study, we characterized alterations in the innate immune system after chronic social defeat stress. Specifically, we determined the phenotype and cytokine producing capacity of various subsets of innate immune cells in stress susceptible and resilient mice. Our data indicate that changes in the myeloid cell compartment involving DCs as well as peripheral and brain invading inflammatory Ly6C^{hi} monocytes are specifically associated with stress susceptibility and resilience based on stress-induced social avoidance. Quantification of DCs in the spleen revealed reduced percentages of these cells in defeated mice, regardless of susceptibility or resilience. Susceptible mice exclusively showed an enhanced maturation phenotype of DCs with elevated expression of MHC class II and co-stimulatory CD80 molecules. In addition, they exhibited higher percentages of TNF-producing CD11b$^+$ cells in the spleen. Susceptibility to stress, but not resilience, was further associated with an increase in inflammatory Ly6C^{hi} monocytes in the spleen and enhanced recruitment of these cells to the brain.

DCs process and present antigens to T cells and therefore represent the main interface between the innate and the adaptive immune response (Takagi et al., 2011; Fukaya et al., 2012; Steinman, 2012). Alterations in the maturation phenotype of splenic DCs characterized by increased levels of MHC class I, CD80 and CD44 molecules have previously been reported in mice after 6 days of repeated social disruption stress (Powell et al., 2009). This altered DC phenotype may be caused by recognition of damage-associated molecular pattern molecules (DAMPs). DAMPs act as endogenous danger signals and alert the innate immune system in response to stress (Fleshner et al., 2017; Franklin et al., 2017). Our data now demonstrate that only susceptibility but not resilience to social stress impacts DC maturation, at least at the level of surface marker expression.

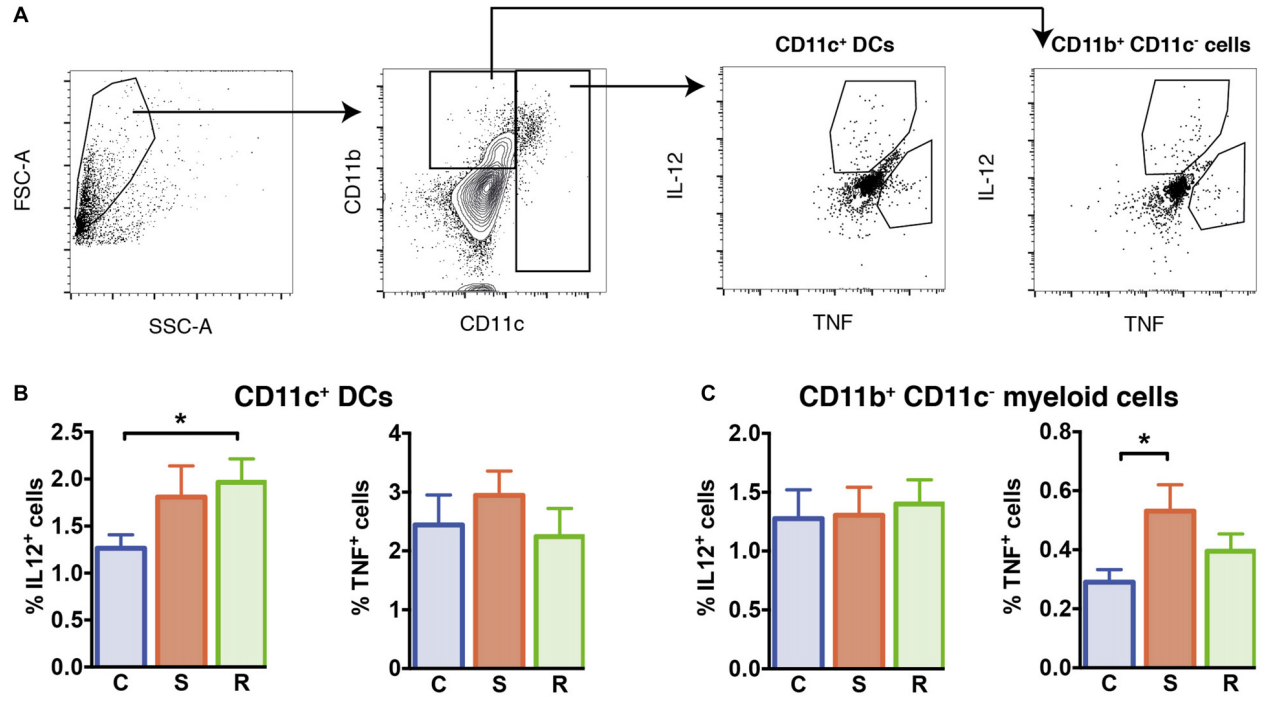

FIGURE 4 | Cytokine production in LPS stimulated isolated splenocytes. **(A)** Gating of interleukin (IL)-12$^+$ and tumor necrosis factor (TNF)$^+$ CD11c$^+$ DCs and CD11b$^+$ CD11c$^-$ myeloid cells. **(B)** Significantly increased percentages of IL-12$^+$ cells in resilient mice among the CD11c$^+$ DCs and similar percentages of TNF$^+$ DCs. **(C)** Similar percentages of IL-12$^+$ cells and increased percentages of TNF$^+$ cells among the CD11b$^+$ CD11c$^-$ myeloid cells in susceptible mice. Bar graphs represent mean + SEM. C, control; S, susceptible; R, resilient. $n_C = 15$, $n_S = 14$, $n_R = 14$. *$p < 0.05$.

These phenotypic alterations specifically occurred in the subset of conventional CD11b$^+$ CD11c$^+$ DCs. In susceptible mice, however, phenotypic changes did not translate to an augmented IL-12 production by these cells. Instead, resilient, but not susceptible animals, displayed an increased proportion of IL-12-producing cells in spleen-derived DCs upon LPS stimulation. IL-12 is known to control the differentiation of naïve CD4$^+$ T cells into T helper 1 cells (Trinchieri, 1995; Watford et al., 2003), which play an important role in adaptive immune responses and have also been implicated in stress responses (Dong and Flavell, 2001; Watford et al., 2003). This "split phenotype" of DCs in resilient and susceptible animals may reflect yet unknown distinct functional properties of these cells associated with differences in behavioral responses to social stress.

In our study, absolute numbers of DCs were not affected by social defeat. However, since numbers of other non-DC cell populations such as neutrophils and other CD11b$^+$ cells increased in the spleen, DC proportions were reduced in both, susceptible and resilient mice. This relative reduction in the DC compartment might compromise their antigen presentation or immunoregulatory properties (Abe et al., 2003; Strother et al., 2016). On the other side, it is possible that enhanced DC maturation augments T cell responses in susceptible mice (Takagi et al., 2011; Fukaya et al., 2012). This has been shown in a model of repeated social disruption. Herein, adoptive transfer of DCs from stressed mice that displayed an enhanced maturation phenotype conferred enhanced adaptive immunity against influenza A virus to recipient animals (Powell et al., 2011). In addition, a plethora of studies supports a role for various CD4$^+$ T cell subsets, particularly pathogenic Th17 cells and T regulatory cells, in stress-induced behaviors in mice (Kim et al., 2012; Beurel et al., 2013; Hong et al., 2013). Furthermore, altered numbers or ratios of these T cell subsets are associated with stress-relevant disorders such as MDD (Chen et al., 2011; Grosse et al., 2016). In this context, we further observed a larger proportion of DCs among peripheral myeloid cells that invaded the brain in both, susceptible and resilient mice. In models of neuroinflammation and CNS autoimmunity, we and others have previously demonstrated a prominent role of DCs in the re-activation of primed T cells upon CNS entry and in local maintenance of Th17 cells (Goverman, 2009; Ransohoff and Cardona, 2010; Poppensieker et al., 2012; Ruland et al., 2017; Scheu et al., 2017). These findings underscore the need to characterize DC-mediated T cell responses in stress susceptibility and resilience after social defeat in future studies. In addition, the effect of CNS invading DCs on CNS resident microglia, astrocytes, and neurons in this model is yet unknown.

In contrast to DCs, neutrophil numbers were equally increased in susceptible and resilient animals. Our findings are in accordance with earlier studies in humans and rodents demonstrating stress-induced enhancement of neutrophil counts (Engler et al., 2004a,b; Heidt et al., 2014; Lafuse et al., 2017). Neutrophils orchestrate the early immune response and react immediately to a pathogenic assault via utilizing

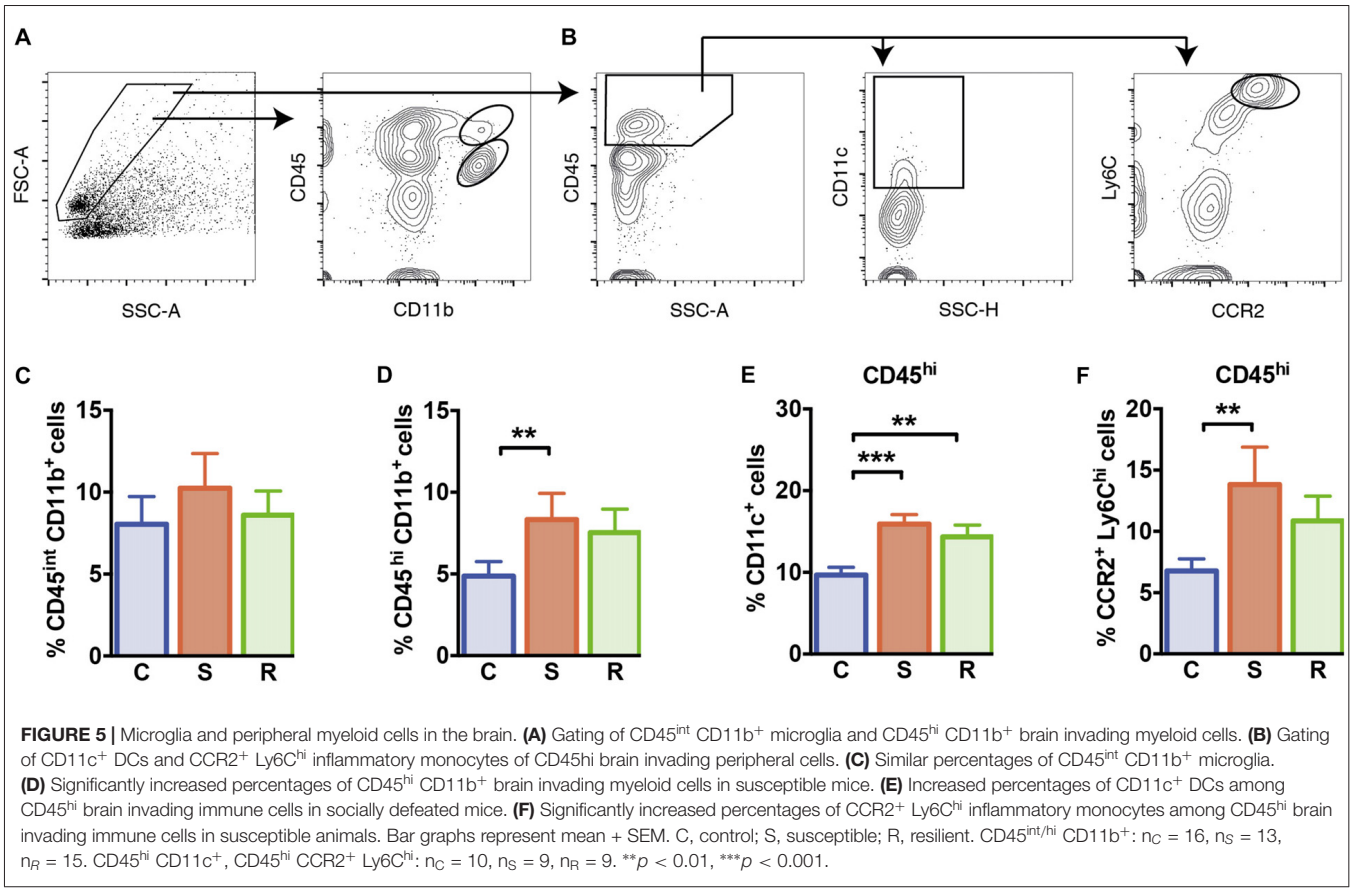

FIGURE 5 | Microglia and peripheral myeloid cells in the brain. **(A)** Gating of $CD45^{int}$ $CD11b^{+}$ microglia and $CD45^{hi}$ $CD11b^{+}$ brain invading myeloid cells. **(B)** Gating of $CD11c^{+}$ DCs and $CCR2^{+}$ $Ly6C^{hi}$ inflammatory monocytes of CD45hi brain invading peripheral cells. **(C)** Similar percentages of $CD45^{int}$ $CD11b^{+}$ microglia. **(D)** Significantly increased percentages of $CD45^{hi}$ $CD11b^{+}$ brain invading myeloid cells in susceptible mice. **(E)** Increased percentages of $CD11c^{+}$ DCs among $CD45^{hi}$ brain invading immune cells in socially defeated mice. **(F)** Significantly increased percentages of $CCR2^{+}$ $Ly6C^{hi}$ inflammatory monocytes among $CD45^{hi}$ brain invading immune cells in susceptible animals. Bar graphs represent mean + SEM. C, control; S, susceptible; R, resilient. $CD45^{int/hi}$ $CD11b^{+}$: $n_C = 16$, $n_S = 13$, $n_R = 15$. $CD45^{hi}$ $CD11c^{+}$, $CD45^{hi}$ $CCR2^{+}$ $Ly6C^{hi}$: $n_C = 10$, $n_S = 9$, $n_R = 9$. $^{**}p < 0.01$, $^{***}p < 0.001$.

various cytotoxic mechanisms (Kobayashi and DeLeo, 2009). Functionally, the increase in neutrophil numbers could serve as adaptive response to stress. This neutrophil-mediated response may prepare the organism for dangerous situations which involve an increased risk for injuries or infections (Dhabhar, 2013). Until now, the function of neutrophils and their potential involvement in stress-induced behavioral changes are not yet fully understood.

In our study, specifically susceptible mice exhibited an increased proportion of $CD11b^{+}$ monocytes that produced proinflammatory TNF after *in vitro* stimulation with LPS. It has frequently been shown that stress exposure results in activation of an inflammatory immune response in mice. For instance, increased blood levels of pro-inflammatory cytokines such as TNF, IL-1β, or IL-6 have been reported after chronic mild stress, social defeat or learned helplessness (Grippo et al., 2005; Hodes et al., 2014; Yang et al., 2015). Our data are further in accordance with earlier studies demonstrating that social defeat stress is associated with higher TNF production by LPS stimulated cultured splenocytes (Kinsey et al., 2008). Various studies have further reported enhanced levels of TNF and other proinflammatory cytokines in the blood and/or cerebrospinal fluid of patients suffering from MDD (Dantzer et al., 2008). Mechanistically, it is thought that peripheral proinflammatory cytokines reach the brain via distinct humoral, neural, and cellular pathways and locally induce detrimental microglia responses (Reyes et al., 1999; Banks, 2005; Capuron and Miller, 2011). Consecutively, activated microglia release proinflammatory cytokines and neurotoxic factors that negatively affect CNS cell functions and thus trigger anxiety- and depression-like behaviors in mice (Reader et al., 2015; Lehmann et al., 2016; Ramirez et al., 2017; Stein et al., 2017). In accordance, earlier studies demonstrated that treatment with TNF induced a depressive-like state in mice (Kaster et al., 2012). TNF application further mediates activation of microglia (Qin et al., 2007), which may result in a self-perpetuating loop of neuroinflammatory disturbance resulting in depression-like behavior. In murine stress models, TNF has also been reported to upregulate indoleamine 2,3-dioxygenase (IDO) implicated as biological mediator of inflammation-related mood disorders (Liu et al., 2015). TNF release has finally been functionally involved in the trafficking of immune cells into the brain during social defeat stress that may locally contribute to production of TNF and other proinflammatory cytokines (Wohleb and Delpech, 2017). In accordance, a recent study determined that upregulation of TNF was associated with higher numbers of Iba-1^{+} microglia in the prefrontal cortex of stress susceptible animals (Couch et al., 2013). Interestingly, we also observed enhanced migration of a subset of $CD11b^{+}$ $Ly6C^{hi}$ monocytes into the brain of susceptible animals, albeit the role of TNF in this process is still unclear.

In addition, $CD11b^{+}$ $Ly6C^{hi}$ monocytes have been shown before to express an inflammatory functional phenotype

(Gordon and Taylor, 2005). We demonstrated that numbers of this monocyte subset increased in the spleen and brain of susceptible mice. This is in line with previous studies showing enhanced numbers of CD11b^{+} Ly6C^{hi} inflammatory monocytes after stress exposure (Wohleb et al., 2013; Heidt et al., 2014; Zheng et al., 2016; Lafuse et al., 2017). Bone marrow-derived myeloid cells have also been shown before to enter the brain of mice that have been stressed by repeated social disruption or foot shocks (Wohleb et al., 2011; Ataka et al., 2013). The recruitment of circulating CD11b^{+} Ly6C^{hi} monocytes into the CNS requires signaling via fractalkine receptor CX_3CR1 and CC chemokine receptor 2 (CCR2), the cognate receptor for CC chemokine ligand 2 (CCL2; Wohleb et al., 2013). Deficiency in CX_3CR1 or CCR2, did not affect the increase of Ly6C^{hi} monocytes in the blood after social disruption stress, but prevented brain infiltration of CD45^{hi} CD11b^{+} macrophages and the development of stress-induced anxiety (Wohleb et al., 2013). It was further shown, that treatment of mice with the natural ginsenoside Rg1 reduced the proinflammatory potential of Ly6C^{hi} monocytes and suppressed their recruitment to the brain via inhibition of CCL2-induced signaling pathways after LPS challenge (Zheng et al., 2016). In consequence, Rg1 treatment resulted in antidepressant effects and reduced social avoidance and anxiety-like behavior after social defeat stress (Zheng et al., 2016). These and other findings in mice showing brain infiltration of Ly6C^{hi} or CD11b^{+} monocytes after 10 days of social defeat stress suggest that brain invasion of these cells is necessary to induce stress-induced behavioral effects (Zheng et al., 2016; Menard et al., 2017). However, these studies did not distinguish between susceptible and resilient mice regarding peripheral myeloid cells in the brain. In our experiment resilient and susceptible animals showed a similar proportion of CD45^{hi} CD11b^{+} peripheral myeloid cells that immigrated into the brain. In contrast, only susceptible mice exhibited enhanced recruitment of Ly6C^{hi} inflammatory monocytes to the brain. These data suggest that brain invading Ly6C^{hi} monocytes may differentially affect behavior in response to stress. As suggested before, these cells may represent an effector cell type involved in promoting behavioral effects in susceptible animals (Wohleb et al., 2013; Zheng et al., 2016).

It is well established, that manipulation of the immune response before induction of a stress response, e.g., by depletion or adoptive transfer of Th17 cells (Beurel et al., 2013), adoptive T cell transfers from stress-exposed donor mice into non-stressed recipients (Brachman et al., 2015), stressor-combined LPS application (Couch et al., 2016), or immunization with heat-killed preparations of microorganisms (Reber et al., 2016) may alter stress vulnerability. Also exposure of mice to distinct gut pathobionts may affect individual variability in susceptibility to stress-associated pathologies (Langgartner et al., 2017). In sum these findings underscore the essential role of altered immune responses for the behavioral reaction to stress. However, it is an open question, whether immune signatures observed in our study harbor predictive potential for stress susceptibility or resilience. We performed endpoint analyses associated with the social avoidance phenotype after stress exposure. Future studies will determine whether a differential immune reactivity before or immediately after onset of social defeat can predict stress vulnerability.

In summary, our findings demonstrate that innate immune alterations occur in response to stress and that stress susceptibility in this model is specifically associated with distinct immune patterns. Susceptible, but not resilient mice exhibit an enhanced DC maturation phenotype, activation of peripheral monocytes, and increased brain recruitment of inflammatory monocytes. Further studies will unravel the influence of the observed DC signature on T cell responses, and the impact of an altered innate immune response on neuronal functions. Together, our findings contribute to a better understanding of immune alterations in stress susceptibility and resilience and their relation to stress-induced behavioral changes.

AUTHOR CONTRIBUTIONS

OA, SS, VA and JA designed the study and the experiments. OA and CR performed the experiments. OA and JA analyzed the data and wrote the first draft of the manuscript. All authors contributed to the revision of the manuscript, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00141/full#supplementary-material

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Psychosocial Stress and Immunity—What Can We Learn From Pig Studies?

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Psychosocial stress may impair immune functions and provoke the development of pathologies. The underlying communication between the brain and the immune system is being studied predominantly in rodents. However, pigs offer several advantages as preclinical models for humans because pigs are more similar to humans than rodents in many anatomical and physiological characteristics. Unlike in rodents, the main stress-induced glucocorticoid in humans and pigs is cortisol with a similar circadian rhythm. In this study, we summarize data on short-term and long-term effects of social stress in pigs for their immunity and neuroendocrine regulation with consequences for their health and well-being. As typical social stressors, regrouping, crowding, social isolation, and maternal deprivation have been studied. Psychosocial stress in pigs may affect various reactions of innate and adaptive immunity, such as leukocyte distribution, cytokine secretion, lymphocyte proliferation, and antibody production as well as immune responses to viral infection or vaccination. Furthermore, social stress may induce or promote gastrointestinal diseases through dysregulation of inflammatory processes. In piglets, psychosocial stress may also result in glucocorticoid resistance of lymphocytes, which has been discussed as a cause of allergic asthma in humans. Stress-related neuroendocrine alterations in the cortico-limbic structures, such as the prefrontal cortex, amygdala, hippocampus and hypothalamus, have been demonstrated in pigs at different ages. Based on these data, we propose using pigs as models for psychosocial stress in humans to study the mechanisms of brain-to-immune and immune-to-brain communication from the systemic level down to the cellular and subcellular levels.

Keywords: social stress, immunity, inflammation, neuroendocrine regulation, *Sus scrofa*

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INTRODUCTION

There is growing evidence that psychosocial stress may affect the immune system in humans. Investigation of the underlying mechanisms of this brain-to-immune communication has been restricted by ethical considerations, which is why animal models are being employed. While rodent models are commonly used, they might not be ideal. Pigs are more similar to humans than rodents in many of their anatomical and physiological characteristics. Therefore, they are used as models for the study of cardiovascular diseases, in gastrointestinal, and pharmacological research as well as in xenotransplantation (Swindle and Smith, 1998; Ekser et al., 2015; Gonzalez et al., 2015; Wyns et al., 2015; Kalder et al., 2016; Wirthgen et al., 2016). Furthermore, the immune system of pigs is

very similar to that of humans in terms of anatomy, function, and gene expression (Freeman et al., 2012; Meurens et al., 2012; Conrad and Johnson, 2015). It has been shown for several immune parameters that the degree of similarity between humans and pigs is significantly higher than the similarity between humans and mice (Dawson, 2011). In addition, pigs are used in neuroscience research because their brain anatomy and development are closer to those of humans than rodents' are (Holm and West, 1994; Lind et al., 2007).

Because of these similarities in immune system and neurobiology of pigs and humans, pigs provide a good model to study immunomodulation by psychosocial stress. It was shown that psychosocial stress in pigs may affect the immune system by alterations in cellular and humoral immune responses, vaccination reactions and glucocorticoid sensitivity. These stress-induced effects are regulated by neuroendocrine mechanisms, which involve the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic-adrenomedullary (SAM) axis, and the limbic system of the brain. There are indications that the HPA axis of pigs more closely resembles the human HPA axis than the axis in rats does. For example, piglets, like human neonates, have no ontogenetic phase in which they are hyporesponsive to stress or adrenocorticotropic hormone (ACTH) challenge (Kanitz et al., 1999), while neonatal rats hardly respond to stress from the age of postnatal day two until their second week of life (Sapolsky and Meaney, 1986). Nevertheless, there is expansive literature on early life stress in rodents reporting effects that are similar to those observed in humans (reviewed in: Bartolomucci, 2007; Hawkley et al., 2012). There are also species differences in circadian rhythms of glucocorticoids. While the circadian rhythm in pigs resembles the rhythm found in humans (Ruis et al., 1997), mice and rats have a circadian rhythm that is opposed to that of humans and pigs (Halberg et al., 1958; Jozsa et al., 2005).

Based on published data, we would like to demonstrate that pigs are excellent animal models to study the effects of psychosocial stress on immune functions and their underlying mechanisms.

SOCIAL STRESS IN PIGS

Pigs are social animals that prefer living in groups with well-established social structures and dominance hierarchies. They strongly perceive social stress when these structures are disrupted. However, management procedures in pig husbandry often do not adequately consider the social needs of the animals and their social bonds. For example, repeated regrouping of pregnant sows represents a social stressor that may affect stress regulation and the immune systems of their offspring (Couret et al., 2009a,b; Otten et al., 2010; Sandercock et al., 2011), with stress in late pregnancy apparently being more relevant for the immune functions of offspring than stress in early pregnancy (Otten et al., 2015). Another important stressor in pigs is the weaning process, which besides environmental and nutritional changes has a strong psychosocial component. The piglets face a sudden and permanent maternal deprivation and regrouping with conspecifics (Campbell et al., 2013). To study the psychosocial stress of weaning, maternal deprivation and isolation have been thoroughly investigated in terms of stress-response regulation and immunomodulatory effects in different experimental models. Regrouping with unfamiliar conspecifics, which is typically performed several times in the lives of domestic pigs, disrupts established social structures. Increased basal salivary cortisol concentrations and a behavior indicating alertness were observed in repeatedly regrouped pigs (Coutellier et al., 2007). Loss of rank in a dominance hierarchy has been shown to be a severe stressor in pigs (Tuchscherer et al., 1998; Otten et al., 1999, 2002). Furthermore, it was shown that social disruption in pigs may affect immune responses in different ways (Hessing et al., 1994; Morrow-Tesch et al., 1994; Deguchi and Akuzawa, 1998; Tuchscherer et al., 1998; de Groot et al., 2001; Ruis et al., 2001; Rudine et al., 2007; Sutherland et al., 2007; Bacou et al., 2017).

The detailed information is summarized in **Table 1** and will be discussed in the next chapter.

IMPACT OF SOCIAL STRESS ON IMMUNITY AND INFLAMMATION

Changes in Innate Immunity

Many studies have investigated social stress effects on leukocyte composition and innate immunity in pigs. Maternal social stress caused attenuated inflammatory responses to challenges in piglets (Sandercock et al., 2011) and reduced the number of circulating leukocytes as well as the CD4+/CD8+ T cell ratio in the offspring (Couret et al., 2009a). Weaning stress potentiated the neuroendocrine response to lipopolysaccharide (LPS) injection as a model for a bacterial infection and induced higher intensity sickness behavior, whereas plasma tumor necrosis factor (TNF) concentrations remained unaltered (Kanitz et al., 2002).

To explicitly study psychosocial stress, a repeated daily isolation procedure of piglets was performed. This isolation stressor diminished TNF increases after LPS challenge and enhanced signs of sickness, which resulted in a stronger relationship between duration of sickness symptoms and physiological measures (Tuchscherer et al., 2004, 2006). This outcome indicates that adaptive responses to immune challenges or disease are sensitized through early isolation. Piglets that experienced a single social isolation for 4 h displayed age-dependent decreases in plasma TNF concentrations and CD4+/CD8+ T cell ratios as well as diminished cytokine release in LPS-stimulated whole blood cultures (Tuchscherer et al., 2009). Furthermore, it was shown that the neutrophil/lymphocyte (N/L) ratio was increased in weaned piglets (Puppe et al., 1997) and in regrouped gilts after social defeat (Ruis et al., 2001).

Other studies document the effects of social stress on the capability to fight infections. Sows that were transferred from group to individual housing activated the innate immune system, as shown by the increased expression of haptoglobin and C-reactive protein (Marco-Ramell et al., 2016). Acutely stressed

TABLE 1 | Social stress effects on hypothalamic-pituitary-adrenocortical (HPA) axis, sympatho-adrenomedullary (SAM) axis, brain and immune system.

Stressor	Treatment period/challenges	Effects on				References
		HPA activity	SAM activity	Brain neurotransmitters and cytokines	Immune system	
PRENATAL STRESS—EFFECTS ON OFFSPRING						
Regrouping of pregnant sows	Twice a week during early gestation	Adrenal weight ↓	n.d.	n.d.	Immunization ↔ LPS challenge ↔	Couret et al., 2009a,b; Otten et al., 2010
	during late gestation	CBG ↓ Adrenal weight ↑ Cell density cortex ↑ (weaning)	Adrenal weight ↑ Cell density medulla ↑ (weaning)	*hippocampus* 5-HIAA/5-HT ↑ (weaning) NA ↑ (relocation)	Leukocyte numbers ↓ lymphocyte proliferation ↑ TNF (LPS *in vitro*) ↓	
POSTNATAL STRESS						
Social isolation of piglets	PND 3 to 11 (2 h daily)	Cortisol, ACTH ↑ CRH (*hypoth.*) ↓ CRH (*amyg.*) ↑	n.d.	IL-1β (*hippo.*) ↑	Lymphocyte proliferation ↓	Kanitz et al., 2004; Tuchscherer et al., 2004, 2006
	LPS: PND 12	Cortisol, CBG, ACTH ↔		TNF (*hippo.*) ↓	TNF, IgG ↔	
	LPS: PND 56	Cortisol, CBG, ACTH ↔		IL-1β (*hippo.*) ↑	TNF ↓ IgG ↔	
Social isolation of piglets	PND 7, 21, or 35 (4 h)	ACTH, cortisol ↑	n.d.	n.d.	TNF ↓ CD4+/CD8+ ratio ↓ IL-1β, IL-10 (LPS *in vitro*) ↓ Lymphocyte proliferation ↑ GC resistance ↑	Kanitz et al., 2009; Tuchscherer et al., 2009, 2010
Social isolation of piglets with conspecific	PND 7, 21, or 35 (4 h)	ACTH, cortisol, FCI ↓ CBG ↑	n.d.	n.d.	GC resistance ↓ IL-6, TNF (LPS *in vitro*) ↓	Kanitz et al., 2014, 2016; Tuchscherer et al., 2014, 2016
Social isolation of piglets followed by *E.coli* challenge	PND 7, 21, or 35 (4 h)	Cortisol ↑ MR mRNA, 11βHSD-1 mRNA (*spleen*) ↑	n.d.	IL-6 mRNA (*hypoth.*) ↑	IL-6 mRNA (*spleen*), TNF (*spleen*) ↑	Tuchscherer et al., 2018
Social isolation of gilts	PND 180 (5 days)	Cortisol ↑	n.d.	n.d.	Acute phase proteins ↑	Marco-Ramell et al., 2016
Weaning	PND 14 or 28	n.d.	n.d.	n.d.	Lymphocyte proliferation ↓	Blecha et al., 1983
Weaning	PND 28 LPS: PND 30	ACTH, cortisol ↑ CBG ↔	n.d.	n.d.	Lymphocyte proliferation ↓ (weaning)	Kanitz et al., 2002
Weaning	PND 19	CRH, cortisol ↑	n.d.	n.d.	Intestinal barrier ↓	Moeser et al., 2007a
Weaning	PND 15, 18, or 23	CRH, cortisol ↑ Age-dependent	n.d.	n.d.	Mast cell activity ↑ Age-dependent	Smith et al., 2010
Weaning	PND 19 or 28	CRH, cortisol ↑ (PND 19, 28) CRH (*mucosa*) ↑ (PND 19)	n.d.	n.d.	Intestinal barrier ↓ (PND 19) Mast cell activity ↑ (PND 19)	Moeser et al., 2007b
Regrouping of pairs of familiar piglets with unknown piglets	PND 33 (3 days)	Cortisol ↑	n.d.	n.d.	Lymphocyte proliferation ↔	Merlot et al., 2004
Regrouping	PND 45, 3 days after vaccination	Cortisol ↑	Noradrenaline ↑	n.d.	Vaccination ↓	de Groot et al., 2001
Regrouping	PND 42 (1 h)	Cortisol ↑	Noradrenaline ↑	n.d.	Leukocyte numbers ↑ TNF, IL-8 (LPS *in vitro*) ↓	Bacou et al., 2017
Regrouping	PND 84 (3 days)	Cortisol ↔	n.d.	n.d.	Lymphocyte proliferation (dominant pigs) ↑ IgG ↑	Tuchscherer et al., 1998

(Continued)

TABLE 1 | Continued

Stressor	Treatment period/challenges	Effects on				References
		HPA activity	SAM activity	Brain neurotransmitters and cytokines	Immune system	
Regrouping	PND 64, 84, 91	Cortisol ↑	n.d.	n.d.	Lymphocyte proliferation ↓	Deguchi and Akuzawa, 1998
Regrouping, heat, crowding	PND 42 (14 days)	Cortisol ↓	n.d.	n.d.	Lymphocyte proliferation ↑ NK cell cytotoxicity ↑	Sutherland et al., 2006
Regrouping, crowding	PND 70 (7 days)	CRH (*mucosa*) ↑ cortisol ↑	n.d.	n.d.	Intestinal barrier ↓ TNF, IL-1β, IL-10 (*intestine*) ↑	Li et al., 2017
Social defeat	PND 70 (15 min)	ACTH, cortisol ↑	Adrenaline, noradrenaline ↑	n.d.	N/L ratio ↑	Ruis et al., 2001

ACTH, adrenocorticotropic hormone; amyg, amygdala; CBG, corticoid-binding globulin; CRH, corticotropin-releasing hormone; E. coli, Escherichia coli; FCI, free cortisol index; GC, glucocorticoid; hippo, hippocampus; hypoth, hypothalamus; IL, interleukin; LPS, lipopolysaccharide; N/L ratio, neutrophil/lymphocyte ratio; n.d., not determined; PND, postnatal day; TNF, tumor necrosis factor.

piglets, which had been moved to single cages on the day of weaning were less susceptible to a challenge infection with *Staphylococcus aureus* (Larson et al., 1985). It is possible that this isolation stress activated the innate immune system, as shown by Marco-Ramell et al. (2016), and led the pigs to be better equipped to handle the infection. A 1 h regrouping of pigs with unfamiliar conspecifics increased leukocyte numbers in the blood and decreased LPS-induced cytokine secretions in whole-blood assays (Bacou et al., 2017). In a recent study, a cross-sensitization between stress and the immune system has been found in piglets that were isolated once for 4 h and then repeatedly challenged with oral *Escherichia coli* infection. These piglets showed a stronger HPA and pro-inflammatory cytokine response to infection (Tuchscherer et al., 2018).

In general, studies in humans and animals have shown that chronic or repeated stress reduce immune responses whereas a single exposure to stress enhances immunity (Dhabhar, 2008), which is also supported by studies on innate immunity in pigs.

Influences on Adaptive Immunity

Effects of Pre- and Postnatal Social Stress

To study social stress effects on adaptive immune responses, mitogen-induced lymphocyte proliferation has often been employed. For example, repeated regrouping of pregnant sows resulted in increased lymphocyte proliferation in response to mitogens in their offspring (Couret et al., 2009a). The authors speculate that prenatal stress affected the development of regulatory T cells, which control proliferative responses. Additionally, the glucocorticoid control of lymphocyte proliferation could have been affected through a phenomenon called glucocorticoid resistance (see section Glucocorticoid Resistance). In contrast, weaning of piglets suppressed mitogen-induced T lymphocyte proliferation (Blecha et al., 1983; Kanitz et al., 2002). Further, weaning followed by LPS challenge decreased IgG concentrations as a result of hyperactivation of the HPA axis (Kanitz et al., 2002).

In a model of repeated maternal deprivation/isolation, mitogen-induced T- and B-cell proliferation was inhibited 1 day but not 45 days after the isolation period, which indicated there was a transient effect (Kanitz et al., 2004). Furthermore, regrouping of unknown piglets resulted in agonistic behavior and long-lasting suppression of lymphocyte proliferation (Deguchi and Akuzawa, 1998). When regrouping was performed by introducing a pair of familiar piglets into a group of unknown piglets, an increase in salivary cortisol and behavioral changes indicated a stress response. The lymphocyte proliferation, however, was unchanged (Merlot et al., 2004). It could be assumed that regulatory T cells were affected. There are indications on the effects of psychosocial stress on regulatory T cells in humans and mice with contradictory results, which require further investigation (Wieck et al., 2013; Ronaldson et al., 2016; Schmidt et al., 2016).

Role of Social Status

Dominance hierarchies, which exist in many social species, are known to influence stress responses. In humans, the concept of rank is rarely applied. Instead, the socioeconomic status is used in psychoneuroimmunological studies as a variable (Sapolsky, 2005). Surely, the socioeconomic status is important in many aspects for human health. However, a subjectively perceived high social status and the threat of losing it have been shown to induce the strongest cortisol response to a laboratory stressor in humans of comparable socioeconomic status (Gruenewald et al., 2006). In pigs, a number of studies investigated the immunological effects of regrouping, which is accompanied by increasing or decreasing social status for some of the pigs. After regrouping of unfamiliar pigs, mitogen-induced proliferation was higher in dominant than in subordinate pigs. Additionally, it increased in dominant pigs and decreased in subordinate pigs with an increasing number of agonistic interactions, which indicates that the controllability

and predictability are critical for the modulation of immune functions (Tuchscherer et al., 1998). In another study, dominant pigs had a higher cellular immune response and were less susceptible to a virus infection than subordinate pigs (Hessing et al., 1994). In contrast, vaccination responses have been shown to be suppressed in regrouped barrows, which resulted in stronger clinical symptoms after a challenge infection with pseudorabies virus (PRV). Here, the dominant pig seemed to be more affected than the subordinate (de Groot et al., 2001). Clearly more work is needed to understand the role of social status in immune responses and to explain these conflicting results.

In humans, bullying is a strong psychosocial stressor involving the social status of an individual. Björkqvist (2001) suggested that the terms "dominant" and "subordinate" in animal studies have their equivalent in "bully" and "victim" in human studies. However, human studies on immunomodulating effects of bullying are scarce (Haavet et al., 2004; Baldwin et al., 2018). While in humans, bullying is just as prevalent in females as in males, female aggression after regrouping is commonly low in mice and rats (Björkqvist, 2001) but has been demonstrated in adult female rats in specific social situations (Albert et al., 1988; Pittet et al., 2017). As aggression after regrouping can reliably be observed in female pigs (Stookey and Gonyou, 1994; D'Eath and Pickup, 2002), this species could serve as a good model for studying immunomodulatory effects of bullying in an experimental setting.

Gastrointestinal Changes

Psychosocial stress may also play a role in the onset and exacerbation of gastrointestinal diseases in humans and animals. The enteric nervous system is connected to the brain by parasympathetic and sympathetic pathways that are called the gut-brain axis (Bhatia and Tandon, 2005). While regrouping of piglets at an age of 32 days did not affect intestinal wall integrity (Koopmans et al., 2006), early weaning resulted in intestinal epithelial barrier dysfunction that was mediated by mast cell activation (Moeser et al., 2007a,b; Smith et al., 2010). It has been shown that the release of mast cell proteases induced by corticotropin-releasing hormone (CRH) is regulated by the enteric nervous system (Overman et al., 2012). Chronic regrouping/crowding stress in pigs impaired ileal and colonic barrier function, whereby the local pro-inflammatory cytokines TNF, IL-1β, and IL-8 were downregulated and the anti-inflammatory cytokine IL-10 was robustly upregulated (Li et al., 2017). These findings are in contrast to studies in rodents (Reber et al., 2008). Such immunosuppression may result in increased susceptibility to gastrointestinal infections, which has been observed in early-weaned piglets (McLamb et al., 2013). Furthermore, gastric ulcerations in slaughtering pigs have been associated with regrouping stress (Gottardo et al., 2017).

These examples show that the pig may also represent an important translational model for research into the influence of gut microbiota on the gut-brain axis, neuroimmunological processes and mood in the context of stress (Rhee et al., 2009; O'Mahony et al., 2015).

Glucocorticoid Resistance

It is known that acute psychosocial stress may result in glucocorticoid resistance of LPS-stimulated lymphocytes (Rohleder et al., 2003). A single episode of social isolation for 4 h reduced the sensitivity of mitogen-induced lymphocyte proliferation to cortisol in suckling piglets (Tuchscherer et al., 2010, 2014). However, social support from the presence of an age-matched conspecific, particularly a familiar piglet, attenuated the cortisol resistance (Tuchscherer et al., 2014, 2016). Although glucocorticoid resistance is probably an adaptive response that secures immune functions during short-term stress, it carries the risk of uncontrolled inflammation and has been discussed as a cause of allergic asthma in humans (Haczku and Panettieri, 2010).

For details regarding the stress models and immune effects, see **Table 1**.

NEUROENDOCRINE REGULATION OF SOCIAL STRESS

Stress activates neuroendocrine systems, which regulate behavior, metabolism, and immune reactions. The HPA axis is the major anatomical substrate of stress responses. Glucocorticoids, which are the end-product of the HPA activation, are important mediators of stress and exert their complex effects via corticosteroid receptors in specific brain areas, which modulate stress adaptation (de Kloet et al., 1998; De Kloet et al., 2005; Black, 2002). While physiological stressors directly activate the paraventricular nucleus of the hypothalamus, psychological stressors require higher processing involving cortico-limbic structures of the brain, such as the amygdala, hippocampus and prefrontal cortex (PFC) (Herman and Cullinan, 1997; Herman et al., 2005). These structures receive further input from the noradrenergic, serotonergic, and dopaminergic systems (Dunn, 1992; Kabbaj et al., 1996; de Laurentiis et al., 2002).

Changes in the limbic and the HPA systems have been observed in pig studies on regrouping, weaning and isolation stress. An elevated mRNA expression of the glucocorticoid metabolizing enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and an increased serotonin turnover after weaning has been shown in piglets of sows stressed by repeated regrouping during pregnancy. Additionally, neuronal activation in the hippocampus has been demonstrated by increased c-fos mRNA expression and noradrenaline concentrations in these piglets (Otten et al., 2010). Regrouping of sows during pregnancy may also affect the stress sensitivity of offspring according to hypothalamic and amygdala CRH mRNA expression (Jarvis et al., 2006). A previous study showed that the weaning process may reduce glucocorticoid receptor (GR) binding in the hippocampus and amygdala (Kanitz et al., 1998). At the level of gene expression, early weaning reduced mRNA expression of 11β-HSD1 and 2, GR and mineralocorticoid receptor (MR) in the hippocampus, while a brief social isolation for 15 min reduced expression of the same genes in the PFC (Poletto et al., 2006). In contrast, a single social isolation of piglets for 4 h caused elevated stress hormone release and open-field reactivity

associated with increased mRNA expression of corticosteroid receptors and metabolizing enzymes in the hypothalamus and hippocampus, while in the amygdala, the MR mRNA expression was decreased. Interestingly, the elevated c-fos mRNA indicated neuronal activation in the hypothalamus and amygdala (Kanitz et al., 2009). Remarkably, social buffering by conspecifics during maternal deprivation attenuated or prevented stress-induced changes in the PFC, amygdala and hypothalamus (Kanitz et al., 2014, 2016). Further, it has been shown that repeated early social isolation resulted in elevated IL-1β concentrations in the hypothalamus and hippocampus as well as enhanced GR binding in the hippocampus. IL-1β drives sickness behavior and is one of the main mediators of brain-immune system communication. Accordingly, stressed piglets showed rather passive patterns of behavior, which can be interpreted as stress-induced sickness behavior or depression. While hypothalamic CRH was still reduced 7 weeks after isolation, it was increased in the amygdala (Kanitz et al., 2004). These data suggest that psychosocial stress may cause long-term effects in the brain-endocrine-immune system.

Effects of social stress on the expression of stress-related genes and the GR binding in the brains of pigs are summarized in **Table 2**.

LIMITATIONS OF PIG MODELS

It should be noted that psychoneuroimmunological studies in pigs are limited for logistic and scientific reasons. Pigs are large outbred animals with a comparatively low breeding rate. With respect to analytical methods, certain impediments exist: (1) the variety of specific antibodies for pig proteins is limited; (2) immunohistochemical studies on pig brains are difficult because pig brains cannot be easily perfused; (3) as of yet, functional MRI can only be performed in anesthetized pigs, which precludes using it for the investigation of emotional processing; (4) there are only a few transgenic pig models to study the role of specific genes (Aigner et al., 2010; Holm et al., 2016). However, based on the increased acceptance of the pig as a valuable model for biomedical research, these problems will be overcome in the near future.

TABLE 2 | Expression of stress-related genes and glucocorticoid receptor (GR) binding in various brain regions.

Stressor	Treatment period/Challenges	Hypothalamus	Hippocampus	Amygdala	Prefrontal cortex	References
PRENATAL STRESS—EFFECTS ON OFFSPRING						
Regrouping of pregnant sows	Twice in mid- (MG) or late gestation (LG)	CRH mRNA (MG)↑ CRH mRNA (LG) ↔	n.d.	CRH mRNA↑	n.d.	Jarvis et al., 2006
	Restraint (30 min) and isolation (1 h) at PND 60	CRH mRNA↑		CRH mRNA↑		
Regrouping of pregnant sows	Twice a week in late gestation	GR binding ↔	GR binding ↔ 11βHSD-1 mRNA ↑ (weaning) c-fos mRNA ↑ (relocation)	n.d.	n.d.	Otten et al., 2010
POSTNATAL STRESS						
Social isolation of piglets	PND 3 to 11 (2 h daily) LPS: PND 12 or 56	n.d.	GR binding ↑ GR binding ↓	n.d.	n.d.	Kanitz et al., 2004; Tuchscherer et al., 2004
Social isolation of piglets	PND 7, 21 or 35 (4 h)	GR mRNA, 11βHSD-1 mRNA, c-fos mRNA ↑	GR mRNA ↔ 11βHSD-1 mRNA ↑	c-fos mRNA ↑	n.d.	Kanitz et al., 2009
Social isolation of piglets with conspecific	PND 7, 21 or 35 (4 h)	MR/GR mRNA ratio ↑ 11β-HSD2 mRNA ↔	MR/GR mRNA ratio, 11β-HSD2 mRNA, c-fos mRNA ↔	MR/GR mRNA ratio ↑ 11β-HSD2 mRNA, c-fos mRNA ↓	MR/GR mRNA ratio ↑ 11β-HSD2 mRNA ↓ c-fos mRNA ↔	Kanitz et al., 2014, 2016
Social isolation of piglets followed by *E. coli* challenge	PND 7, 21 or 35 (4 h)	11β-HSD1 mRNA, 11β-HSD2 mRNA, IL-6 mRNA ↑	n.d.	n.d.	GR mRNA, 11βHSD-1 mRNA, 11β-HSD2 mRNA ↑	Tuchscherer et al., 2018
Weaning followed by social isolation	PND 10	n.d.	11β-HSD1 mRNA, 11β-HSD2 mRNA, GR mRNA, MR mRNA ↓	n.d.	11β-HSD1 mRNA, 11β-HSD2 mRNA, GR mRNA, MR mRNA ↔	Poletto et al., 2006
	Isolation: PND 12 or 23 (15 min)		11β-HSD1 mRNA, 11β-HSD2 mRNA, GR mRNA, MR mRNA ↔		11β-HSD1 mRNA, 11β-HSD2 mRNA, GR mRNA, MR mRNA ↓	
Weaning	PND 35	GR binding ↔	GR binding ↓	GR binding ↓	n.d.	Kanitz et al., 1998

11β-HSD, 11β-hydroxysteroid dehydrogenase; CRH, corticotropin-releasing hormone; E. coli, Escherichia coli; IL, interleukin; LPS, lipopolysaccharide; MR, mineralocorticoid receptor; n.d., not determined; PND, postnatal day.

CONCLUSIONS AND FUTURE DIRECTIONS

Psychosocial stress in pigs has often been studied in experimental setups that are similar to common practices in animal husbandry. Although these stressors usually do not have equivalents in human life, they may reflect the moderate intensity of psychosocial stress in the human society better than some of the rodent stress models. Interestingly, pig studies show that social rank and agonistic interactions to achieve or defend rank have a substantial influence on immune responses. The findings strongly suggest investigating immunomodulatory social stress effects in humans based on their social rank. The main advantage of pig models is to gather information, which is not available from human studies due to ethical reasons. For example, prenatal and early postnatal stress models in pigs may reveal developmental alterations in neuroendocrine stress regulation and immunity with relevance for humans. Present limitations of pig models are counterbalanced by the opportunity to perform complex investigations of behavioral, neuroendocrine, and immunological interactions in a species, which is not only physiologically highly similar to humans but also in its emotionality and social behavior. Despite of these advantages, we would like to emphasize that both, rodent and pig models have their justifications. Combining their specific strengths may boost psychoneuroimmunological research into social stress in humans.

AUTHOR CONTRIBUTIONS

UG, MT, and EK contributed to the conception and the design of the perspective. UG wrote the manuscript. MT and EK revised it critically for important intellectual content.

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REVIEW
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Neuroendocrine Control of Macrophage Development and Function

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Macrophages carry out numerous physiological activities that are essential for both systemic and local homeostasis, as well as innate and adaptive immune responses. Their biology is intricately regulated by hormones, neuropeptides, and neurotransmitters, establishing distinct neuroendocrine axes. The control is pleiotropic, including maturation of bone marrow-derived myeloid precursors, cell differentiation into functional subpopulations, cytotoxic activity, phagocytosis, production of inflammatory mediators, antigen presentation, and activation of effector lymphocytes. Additionally, neuroendocrine components modulate macrophage ability to influence tumor growth and to prevent the spreading of infective agents. Interestingly, macrophage-derived factors enhance glucocorticoid production through the stimulation of the hypothalamic–pituitary–adrenal axis. These bidirectional effects highlight a tightly controlled balance between neuroendocrine stimuli and macrophage function in the development of innate and adaptive immune responses. Herein, we discuss how components of neuroendocrine axes impact on macrophage development and function and may ultimately influence inflammation, tissue repair, infection, or cancer progression. The knowledge of the crosstalk between macrophages and endocrine or brain-derived components may contribute to improve and create new approaches with clinical relevance in homeostatic or pathological conditions.

Keywords: macrophages, monocytes, neuroendocrine system, hormones, neurotransmitters, stress, glucocorticoids

INTRODUCTION

Integration of body functions relies on distinct mechanisms encompassing various organs and systems. As a consequence, perturbations in the environment or in tissue homeostasis usually produce quick and effective responses that may result in significant local and systemic impacts, for example, due to a sustained communication between the central nervous system (CNS) and peripheral organs through the concerted activities of cell type-specific chemical messengers. Major neuroendocrine axes comprise the hypothalamus and the pituitary in the brain signaling to adrenal glands [hypothalamus–pituitary–adrenal (HPA) glands or HPA axis], thyroid [hypothalamus–pituitary–thyroid (HPT) axis], or gonads [hypothalamus–pituitary–gonad (HPG) axis]. They exert regulatory effects on the immune system, and any imbalance disrupting such neuroimmunoendocrine communication may result in pathological conditions (1–3).

Among the cells of the immune system involved in neuroendocrine interactions, macrophages play a central role in the activation and modulation of both innate and adaptive immune responses. Interestingly, an intricate bidirectional macrophage-neuroendocrine system crosstalk is currently being explored to understand homeostasis and diseases. Here, we review how macrophages bridge the immune, endocrine, and nervous systems, how hormones and neurotransmitters may influence their physiology and function and to what extent such circuitry may be placed as potential therapeutic targets in various diseases. We do not extend our analysis on how macrophage-derived mediators affect brain activity and behavior, since recent comprehensive reviews about this issue have been published (4–6).

MACROPHAGES AND NEUROENDOCRINE COMPONENTS

Macrophages are multifunctional leukocytes that recognize and remove invading pathogens, toxins, cellular debris and apoptotic cells in healthy or inflamed tissues. They are tissue-resident cells that have settled during embryogenesis or monocyte-derived cells that migrated from the blood circulation and reached different organs (7). Depending on the organ they populate, macrophages receive different designations, which are supported by specific differentiation programs, cell morphologies, and specialized functions (8, 9). To name a few, they are known as microglia in the brain, alveolar macrophages in the lungs, Kupffer cells in the liver, osteoclasts in bones, and chondroclasts in cartilages. More specifically, macrophages are further classified into distinct subpopulations based on their functional properties, which may comprise non-activated circulating monocytes, pro-inflammatory or anti-inflammatory macrophages, among others. Indeed, strong plasticity in their differentiation and the notion that these subsets may be interchangeable have led to the proposition of a "spectrum wheel" system (10–12). In this regard, additional cellular subsets can be defined by a combination of characteristics, as it will be discussed below.

To perform their roles, macrophages rely on a wide range of specific surface and intracellular receptors. These sensors are able to recognize microbial components, defined as pathogen-associated molecular patterns, and danger molecules released after cell and tissue lesions, defined as damage-associated molecular patterns. Macrophages activated through these receptors produce potent pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-12, together with chemokines and toxic-free radicals (13–15). Furthermore, other receptors play critical roles on macrophage function. For example, scavenger receptors (SRs) bind a diverse range of ligands from bacteria to native proteins, allowing them to regulate both cell adhesion and removal of noxious agents by phagocytosis (16). A class of SRs includes the mannose receptor (CD206), which is a marker of M2 macrophages (see below). In fact, when CD206-positive macrophages were eliminated from the lungs of a murine transgenic model of toxemia by the administration of diphtheria toxin, mice developed an exacerbated lung inflammation upon endotoxin challenge (17). Death of neighboring cells by apoptosis is initially perceived by macrophages through specific receptors that recognize phosphatidylserine exposure in the lipid bilayer membrane of dying cells. Macrophages are also able to detect complement molecules through cognate receptors and antibodies through Fc receptors. These molecules opsonize pathogens and abnormal cells, thus stimulating their phagocytosis.

Analyses of a large body of evidence revealed that macrophages could respond to a wide variety of neuroendocrine factors [e.g., Ref. (18)]. In particular, a second-level evaluation of published data sets available through the Immunological Genome Project (ImmGen) Consortium (19) shows that monocytes and macrophages express not only numerous hormone and neurotransmitter receptors (**Figure 1**) but also a number of the corresponding cognate ligands. More specifically, using neuroendocrine-related gene expression profiles, it is possible to cluster myeloid cells by cell type and body locations (Figure S1 in Supplementary Material). Accordingly, the genes for angiotensin I converting enzyme (*Ace*), 15-hydroxyprostaglandin dehydrogenase (*Hpgd*), and EGF-like module containing, mucin-like, hormone receptor-like sequence 4 (*Emr4*/*Adgre4*) are highly expressed in different populations of circulating monocytes. Contrasting with this transcriptomic observation, Stacey et al. (20) have identified higher expression levels of *Emr4*/*Adgre4* predominantly in resident macrophages. In a second hand, genes such as *Ltc4s*, *Ptgs1*, *Igf1*, *Ophn1*, *Hpgds*, *Gatm*, *Pgcp*, and *Cysltr1* were highly expressed in most macrophages and some populations of dendritic cells, but slightly expressed in monocytes. Together, the presence of neuroendocrine components in monocytes and macrophages provide the grounds for the notion that macrophage-neuroendocrine crosstalk influences the overall homeostasis and immunity of an individual.

In the sections below, we will discuss in more detail how hormones, nervous-derived cytokines, and neurotransmitters regulate different aspects of macrophage biology related to the preservation of internal homeostasis.

NEUROTRANSMITTERS AND HORMONES REGULATE MACROPHAGE FUNCTION

The vast number of neuroendocrine factors places a significant challenge for the quest to unravel brain-immune communication. Nevertheless, it may also unveil numerous possibilities for clinical intervention. The early isolation of specific hormones and the availability of recombinant proteins, as well as gene editing technologies, have allowed the study of various molecules of interest in macrophage physiology. The first studies showing that macrophages were able to respond to neurotransmitters date back to mid-past century with the finding that phagocytosis was stimulated by histamine (21). This small monoamine messenger is produced by some immune cells (e.g., mast cells and basophils) and by neurons of the tuberomammillary nucleus of the hypothalamus (22, 23). The biological significance of histamine to macrophage function was later demonstrated in distinct models of intracellular infection (24–26) and paved the way for the investigation of other neurotransmitters endowed with similar properties to modulate macrophage physiology.

The discovery that macrophages also respond to hormonal stimuli came shortly after. Then, a large body of publications

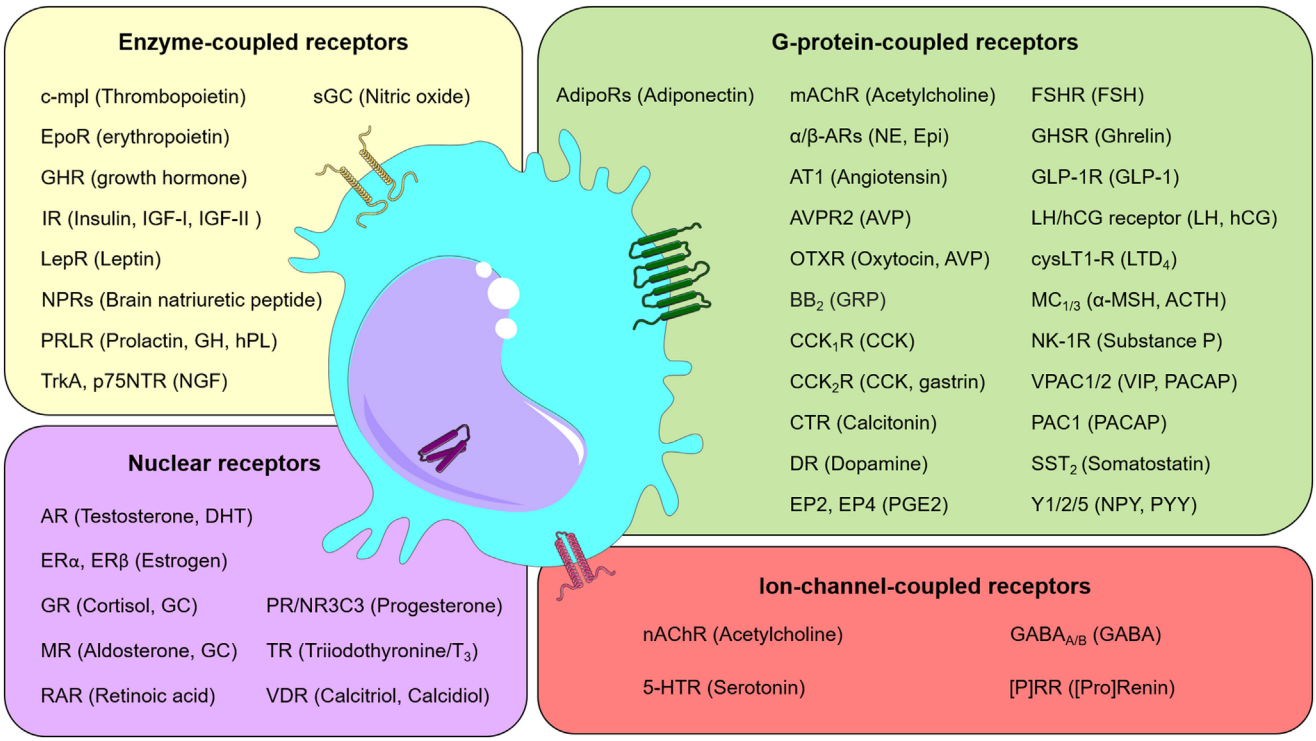

FIGURE 1 | Neuroendocrine communication on macrophages. Schematic representation listing selected receptors (and their ligands) found in macrophages. Receptors were grouped into classes, as indicated. Abbreviations: (P)RR, (pro)renin receptor; 5-HTR, serotonin receptor; ACTH, adrenocorticotropic hormone; AdipoRs, adiponectin receptors; AR, androgen receptor; AT1, angiotensin II receptor type 1; AVP, arginine vasopressin or antidiuretic hormone; AVPR2, arginine vasopressin receptor 2; BB2, bombesin receptor; CCK, cholecystokinin; $CCK_{1/2}R$, cholecystokinin receptor 1/2, respectively; c-mpl, myeloproliferative leukemia protein; CO, carbon monoxide; CTR, calcitonin receptor; cysLT1-R, cysteinyl leukotriene receptor 1; DHT, dihydrotestosterone; DR, dopamine receptor; EP2, prostaglandin E2 receptor 2; EP4, prostaglandin E2 receptor 4; Epi, epinephrine; EpoR, erythropoietin receptor; ER, estrogen receptor; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; GABA, gamma-aminobutyric acid; $GABA_{A/B}$, $GABA_A$-receptor and $GABA_B$-receptor, respectively; GC, glucocorticoids; GH, growth hormone; GHR, growth hormone receptor; GHSR, growth hormone secretagogue receptor (also known as ghrelin receptor); GLP-1, glucagon-like peptide-1; GLP-1R, Glucagon-like peptide-1 receptor; GR, glucocorticoid receptor; GRP, gastrin-releasing peptide; hCG, human chorionic gonadotropin; hPL, human placental lactogen; IGF, insulin-like growth factor; IR, insulin receptor; LepR, leptin receptor; LH, luteinizing hormone; LTD_4, leukotriene D_4; mAChR, muscarinic acetylcholine receptor; $MC_{1/3}$, melanocortin 1/3 receptor, respectively; MR, mineralocorticoid receptor; nAChR, nicotinic acetylcholine receptor; NE, norepinephrine; NGF, nerve growth factor; NK-1R, neurokinin 1 receptor; NPRs, natriuretic peptide receptors; NPY, neuropeptide Y; NR3C3, nuclear receptor subfamily 3, group C, member 3; OTXR, oxytocin receptor; p75NTR, neurotrophin receptor p75; PAC1, pituitary adenylate cyclase-activating polypeptide type I receptor; PACAP, pituitary adenylate cyclase-activating peptide; PGE2, prostaglandin 2; PR, progesterone receptor; PRLR, prolactin receptor; PYY, Peptide YY; RAR, retinoic acid receptor; sGC, soluble guanylyl cyclase; Soluble guanylyl cyclase (GC-1); SST_2, somatostatin receptor type 2; TR, thyroid hormone receptor; TrkA, transmembrane tyrosine kinase; VDR, vitamin D receptor; VIP, vasoactive intestinal peptide; VPAC1/2, vasoactive intestinal peptide receptor 1/2, respectively; Y1/2/5, neuropeptide Y receptor type 1/2/5, respectively; α/β-ARs, α/β-adrenergic receptors; α-MSH, melanocyte-stimulating hormone.

showed that hormones can broadly modulate both the immune system and inflammatory responses [e.g., Ref. (27–31)]. Among them, early clinical observations and experimental investigations provided evidence that the formation of the so-called granulation tissue was impaired upon treatment with cortisone or adrenocorticotropic hormone (ACTH) (29, 32). The absence of this delimited transitory regenerative response full of macrophages and new blood vessels raised the assumption that corticosteroids could weaken macrophage function, a hypothesis that was later undermined by the finding that corticosteroids were shown to promote macrophage migration in culture (33, 34). Indeed, the effects of corticosteroids on the formation of granulation tissue seem to rely on suppressing blood vessel formation through the inhibition of platelet-derived growth factor-dependent expression of vascular endothelial growth factor (35). Since those discoveries, reports concerning neuroendocrine modulation of the immune system have become available, and many comprehensive reviews have been published (36, 37). Therefore, herein, we will focus on the influence of neuroendocrine messengers on macrophage physiology by dividing the distinct stages of their lifespan into the following sub-sections.

Monocyte/Macrophage Maturation May Be Driven by Neuroendocrine Components

Although macrophage origin remains a matter of intense debate, they seem to arise from at least two distinct locations. Early in

life, *Myb*-independent yolk sac-derived erythro-myeloid Csf1r-positive progenitors emerge from blood islands, colonize the developing liver at early- to mid-gestation, and subsequently reach other organs, such as lungs, epidermis, and brain (9, 38–43). These macrophages can persist and proliferate in either healthy young adults or upon tissue insult (40, 44–46). Later, new macrophages and dendritic cells differentiate from bone marrow-derived circulating monocytes upon reaching a target tissue damaged by inflammatory reactions or pathogens.

The influence of neuroendocrine messengers on early macrophage differentiation is largely underappreciated, but a closer look at the data published by Mass et al. (9) reveals that many neuroendocrine-related genes are differentially expressed during macrophage stepwise maturation (**Figure 2**). Some of them, like calcitonin-related genes (*Calcrl* and *Ramp2*), prostaglandin-associated genes (*Ptgis* and *Ptger4*), and both *Vipr2* and *Ghr* are highly expressed in erythro-myeloid progenitors (EMPs), but gradually decline as cells differentiate into macrophages. The glutamate receptor gene *Gria3* exhibits high expression levels in EMPs, a further increase in $CD45^{+}Kit^{-}Lin^{-}$ pre-macrophages (pMacs), and a subsequent reduction in mature macrophages. In turn, the erythropoietin receptor gene (*Epor*) is detected in intermediate levels either in EMPs or macrophages, but it shows a low expression in pMacs. Other genes, such as *Adbr2* (β2-adrenergic receptor), *Ednrb* (endothelin receptor type B), and *Igf1* (insulin-like growth factor 1), exhibit lower levels in EMPs and pMacs, but higher expression in macrophages. Together, these expression profiles suggest that neuroendocrine signals modulate macrophage maturation and may affect macrophage function. Experimental investigations using selective activation or inactivation of genes of interest in conditional systems are, therefore, necessary to elucidate possible medical benefits from manipulating neuroendocrine influence on macrophage differentiation.

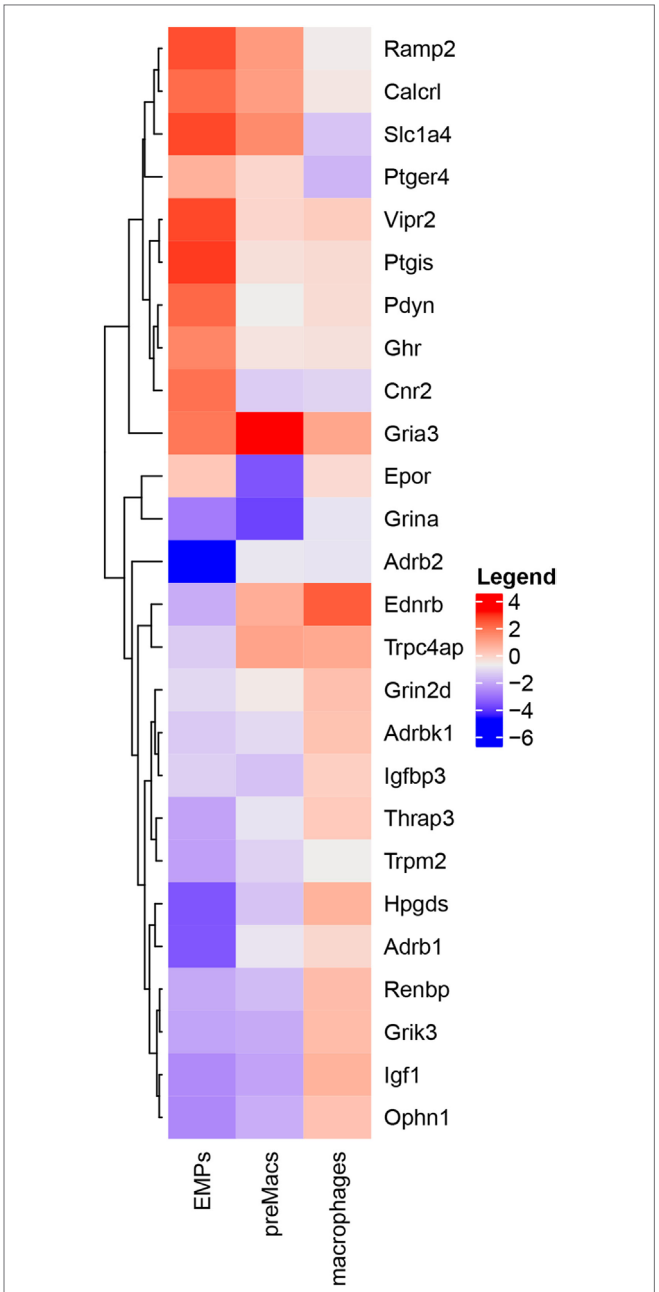

FIGURE 2 | Neuroendocrine-associated genes are differentially expressed during macrophage differentiation. Heatmap of RNA-Seq profile filtered by keywords from Mass et al. (9) depicting hierarchically clustered relative gene expression (log2) in erythro-myeloid progenitors, pre-macrophages (preMacs), and macrophages. Levels of expression are represented by colors in which red, white, and blue indicate high, intermediate, and low intensities, respectively.

Macrophage Polarization in Response to Neuroendocrine Stimuli

It is acknowledged that it is through cell polarization that proper macrophage effector responses can be achieved in target tissues. However, macrophage nomenclature may be confusing to reconcile between *in vitro*-induced phenotypes and their *in vivo* relevance. From now on, we will refer to macrophage subsets based on their functions or cellular markers, thus avoiding whenever possible the misconceptions that may arise from distinct stimulation conditions among laboratories (47).

The preference toward a given functional phenotype of macrophages is classically triggered by cytokines and specific pathogens. Formerly known as M1 or classically activated macrophages, cells involved in host defense against microbes and tumors show a pro-inflammatory phenotype induced by IL-12 and IFN-γ [M(IFNγ) macrophages]. Interestingly, they also participate in the onset of tissue repair upon an injury or trauma (47, 48). In turn, a heterogeneous population of anti-inflammatory macrophages may arise secondary to stimulation with Th2 cytokines, glucocorticoids, immune complexes, colony stimulating factor-1, or by some intracellular microorganisms, such as *Leishmania* (49, 50). Initially associated with wound healing and known as alternatively activated or M2 macrophages, these anti-inflammatory cells show increased arginase-1 activity, which generates ornithine and urea. Ornithine is subsequently converted to proline and polyamines, which are used in the biosynthesis of collagen and in cell proliferation, respectively (51, 52). By converting arginine to ornithine, arginase-1 competes with the nitric oxide (NO)-producing enzyme NO synthase characteristic

of pro-inflammatory macrophages (53). Other anti-inflammatory macrophages may be produced by the incubation with IL-10 [M(IL-10)] or glucocorticoids and TGF-β [M(GC + TGFβ)], thereby polarizing them to a pro-healing phenotype with high scavenging activity also known as "deactivated, regulatory, or M2c macrophages" [reviewed by Martinez et al. (54)]. Unlike M(IFNγ), M(IL-4), or M(IL-10) macrophages, glucocorticoid-induced macrophages express higher levels of Mer tyrosine kinase (MerTK), a surface receptor involved in the phagocytosis of early apoptotic cells through the recognition of exposed phosphatidylserine (55). Despite their specificities, these phenotypes are plastic, and macrophages can switch between distinct functions both *in vivo* and *in vitro* upon a number of distinct stimuli (10–12, 56–58).

The finding that glucocorticoids influence macrophage polarization points out that neuroendocrine components may also contribute to macrophage subset decision and hence tissue regeneration. In this regard, Gratchev et al. (59) have demonstrated that M(IL-4) macrophages secrete extracellular matrix (ECM) components and remodeling enzymes, as tenascin-C and metalloproteases, respectively, whereas M(GC) macrophages exhibited undetected or reduced levels of many ECM-associated proteins. Of note, cell-specific gene inactivation of the mouse glucocorticoid receptor (GR) in the myeloid lineage impaired cardiac healing after experimental ischemic injury, due to abnormal collagen scar formation, reduced neovascularization, and persistent pro-inflammatory differentiation of macrophages. Moreover, dexamethasone can overcome the effects of IL-4 on the production of macrophage-derived ECM molecules. Unlike M(IL-4) cells, only M(IL-4 + dexa) or M(GC) macrophages were responsive to TGF-β, a major cytokine in the resolution phase (**Figure 3**) (60). Thereby, dexamethasone modulates the resolution phase by inhibiting the expression of NF-κB-dependent pro-inflammatory cytokines involved in the initial dominant inflammatory phase while inducing a resolutive and reparative phenotype (61–64). This limits inflammation and highlights the preponderant role of glucocorticoids on the regulation of macrophage function.

The modulatory properties of neurotrophic factors and neuropeptides are also prominent in the regulation of immune cells, including monocytes and macrophages. For instance, norepinephrine by itself is a potent inducer of alternatively activated macrophages even in the presence of LPS (65). By contrast, activation of monocytes and macrophages by LPS induces increased

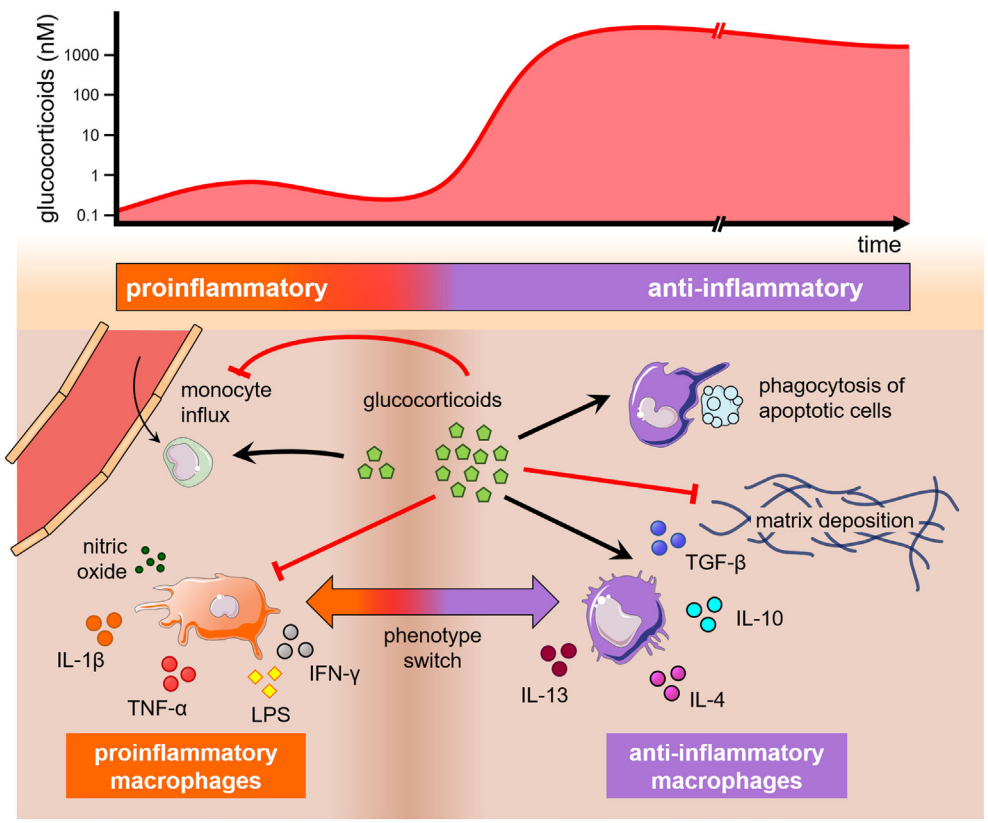

FIGURE 3 | The influence of glucocorticoids on monocytes and macrophages. Glucocorticoids may play opposing effects on monocytes and macrophages depending on theirs levels and time of exposure (top). A graphical representation depicts these effects on monocyte trafficking into tissues, macrophage polarization, and phagocytosis (bottom). A short and low increase in glucocorticoid levels (left) stimulates monocyte extravasation into the injured tissue, while high and long-lasting levels of glucocorticoids (right) inhibit monocyte proliferation and extravasation, as well as drive macrophage polarization into anti-inflammatory phenotypes that may produce extracellular matrix or stimulate the engulfment of apoptotic cells. All these phenomena are also influenced by cytokines present in the milieu.

production of the neurotrophin nerve growth factor (NGF), which favors a pro-inflammatory phenotype through the induction of monocyte cytotoxic potential and the production of TNF-α (66, 67). Importantly, macrophage production of NGF protects them against apoptosis during inflammation or HIV-1 infection (67, 68). However, the protective survival role of NGF in HIV-1-infected macrophages may be counterbalanced by its stimulating effect on viral replication, which occurs through the downregulation of the cytidine deaminase APOBEC3G (69). Neurotrophins may actually play opposing roles in their abilities to control the growth of pathogens in macrophages, since NGF protects macrophage from infection with the protozoan *Leishmania donovani* through the increased production of hydrogen peroxide (70). Meanwhile, the widely distributed vasoactive intestinal peptide and the pituitary adenylate cyclase-activating polypeptide (PACAP) downregulate macrophage-derived production of numerous pro-inflammatory molecules, whereas inducing macrophage synthesis of the anti-inflammatory mediators IL-4 and IL-10 (71–73). Overall, neuropeptides are able to modulate macrophage-dependent activities and promote body homeostasis in conditions as diverse as recovering injured nerve tissue, restraining tumor progression or preventing HIV-1 production (72, 74, 75).

Modulation of Macrophage Migration by Hormones and Neurotransmitters

The movement of cells is central for proper physiology. During macrophage ontogeny in adults, progenitor cells leave the bone marrow-associated hematopoietic stem cell niche and reach the bloodstream, where they can travel to distant parts of the body. Upon any insult, circulating monocytes are recruited to different tissues by specific chemotactic factors, such as CC- and CX_3C-chemokine ligands. The receptors of these chemotactic factors also contribute to defining specific monocyte subsets. In humans, classical monocytes are defined by high expression levels of the LPS co-receptor CD14 and the absence of the Fcγ receptor CD16, while expressing high levels of the CC-chemokine receptor 2 (CCR2) and low levels of the CX_3C-chemokine receptor 1 (CX_3CR1). In turn, $CD16^+$ monocytes can be further divided into two groups, both expressing high levels of CX_3CR1 and low levels of CCR2. Non-classical cells are $CD14^{lo}CD16^{hi}$, whereas intermediate cells are $CD14^{hi}CD16^{lo}$ (76). In this regard, several studies have reported that both hormones and neurotransmitters can modulate monocyte or macrophage migration by tuning the levels of target tissue-derived chemokines or the expression of their corresponding receptors (77–79). This lends an additional level of complexity to the control of macrophage behavior in health and disease.

One of these mechanisms includes the α7 and α9-nicotinic acetylcholine receptor (nAChR)-mediated downregulation of CCL2 expression in the brain of the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis treated with nicotine. The inhibition of brain-derived CCL2 by nicotine impaired the recruitment of pro-inflammatory $CCR2^+Ly6C^{hi}$ monocytes during murine EAE—which play a role similar to that of human classical $CD14^+CD16^-$ monocytes—and could be an alternative for mitigating neuroinflammation in clinical settings (80). In addition, the nAChR-dependent modulation of CCL2 expression may also contribute to treating genetic disorders, such as the Duchenne muscular dystrophy (DMD). This dystrophin-related, X-linked condition is worsened by persistent muscle inflammation, including the respiratory muscles, which can cause patient death. A study using *mdx* mice as a model of DMD found that the deficiency of CCR2 lowered the number of muscle-infiltrating inflammatory monocytes and macrophages, therefore, ameliorating disease severity and improving muscle strength (81). Together, this evidence suggests that modulating the migration of monocytes and macrophages through neuroendocrine components may be a valuable tool to control inflammatory diseases.

STRESS-ASSOCIATED NEUROENDOCRINE MEDIATORS REGULATE MACROPHAGE PHYSIOLOGY

A definition of stress can be troublesome, but usually involves an uncontrolled response of the body to aversive changes that may lead to anxiety, emotional tension, or fear. At the cellular level; however, stress is the result of ACTH release by the pituitary followed by the discharge of glucocorticoids produced by the adrenal cortex, along with the release of norepinephrine and epinephrine by the sympathetic-adrenomedullary (SAM) axis of the autonomic nervous system and the adrenal medulla, respectively (82). Glucocorticoids bind their nuclear GR through a mechanism dependent on GR-interacting protein-1 (GRIP1) phosphorylation by cyclin-dependent kinase-9 (83). Activation of this pathway leads to a broad repression of inflammatory-associated genes regulated by the transcription factors NF-κB and AP1, and an upregulation of anti-inflammatory genes, such as the NF-κB repressor glucocorticoid-induced leucine zipper (*Gilz*) (61–63, 84). In turn, signaling by norepinephrine and epinephrine relies on their interaction with β2-adrenergic receptors (β2-ARs), which are seven-pass transmembrane receptors of the family of G-protein coupled receptors. Upon ligand binding-triggered conformational changes, they couple heterotrimeric G_s proteins that relay signals to adenylyl cyclase for the production of cyclic AMP (cAMP) and subsequent activation of protein kinase A that may translocate to the nucleus and activate cAMP response element-binding protein to ultimately alter the transcription of target genes (85, 86). When any imbalance disrupting such neuroimmunoendocrine communication occurs, individuals are prone to immunosuppression and increased susceptibility to disease. This is a common occurrence nowadays due to the high number of people living under stressful conditions (87).

It is well established that stress-related mediators vastly affect monocytes and tissue-resident macrophages. For instance, the continued administration of glucocorticoids increases the number of monocytes in the periphery and within the bone marrow, while inducing a substantial reduction of the lymphoid population (88, 89). In culture, however, glucocorticoids were previously known to suppress macrophage growth (90). This apparent discrepancy is found in other monocyte/macrophage processes, such as monocyte trafficking. In fact, Rinehart et al.

(91) observed that hydrocortisone succinate impaired the migration of human monocytes in culture, whereas other studies showed that glucocorticoids might stimulate monocyte migration by transiently increasing CCR2 expression in response to moderate physical exercise or transient stress (92, 93). These findings and many others support the notion that drastic changes in glucocorticoid pharmacological activity may occur as their levels and time of exposure increase. Thus, a brief and low-level exposure to glucocorticoids seems to prepare the tissue environment for a greater inflammatory cell response in case of a subsequent insult, whereas sustained high levels of glucocorticoids produce their well known anti-inflammatory properties (**Figure 3**) (93).

Stressors such as prolonged restraining, cold or heat exposure, footshocks, opioid administration, or psychological challenges disturb macrophage phagocytosis in mice (94–97). In general, these conditions activate both the HPA and SAM axes, while diminishing the levels of pro-inflammatory cytokines (98, 99). For example, cold-induced stress impaired the engulfment of apoptotic thymocytes by LPS-activated macrophages through a glucocorticoid-dependent mechanism that was accompanied by an increase in IL-10 levels. By contrast, treatment of INF-γ-activated macrophages [M(IFNγ)] with glucocorticoids enhanced Fc-mediated phagocytosis of sheep red blood cells in culture (100), thus revealing that the cellular context plays a critical role in macrophage responses. A rise of epinephrine and norepinephrine plasma levels also followed the acute cold stress, but these catecholamines seemed to have no influence on phagocytosis (96). Yet, these findings are at odds with other observations. In particular, many studies have shown that treatments with corticosterone or the glucocorticoid analogs methylprednisolone, dexamethasone, or hydrocortisone augmented macrophage phagocytosis (59, 101), an outcome that was not observed for the mineralocorticoid aldosterone or the sex steroids estradiol or progesterone (102). Taken together, the available information points out that the local or systemic release of stress hormones modulates macrophage ability to phagocytose and may exert a significant impact on both innate and adaptive responses, since the engulfment of apoptotic cells leads to the upregulation of anti-inflammatory genes and cytokines by macrophages (103–105).

Upon phagocytosis, macrophages process and present antigens through MHC molecules to T cells, which might differentiate into unique subsets, such as Th1, Th2, and Th17, each of them bearing specific functions. Stressful conditions may affect macrophage antigen presentation and modify the Th1/Th2/Th17 balance by altering the macrophage cytokine profile and thereby increasing susceptibility to infections or allergic processes (2). For instance, a 4-day exposure of mice to cold water lowered the IFN-γ-induced expression of MHC class II molecules in macrophages (106). Similar findings were observed in restrained mice, in which macrophages showed reduced levels of MHC class II and upregulated concentrations of plasma corticosteroids (107, 108). A rise of serum glucocorticoids and a concomitant decrease in the production of NO through the activation of the HPA axis upon acute cold-induced stress result in the development of an immunosuppressive response that is enhanced by norepinephrine-producing fat-resident macrophages (109–111). Likewise, a Th2-immune response could be observed in heat-stressed mice, which exhibited high plasma levels of norepinephrine and increased macrophage production of CCL2, whose synthesis was controlled by norepinephrine depletion (112). Glucocorticoids, norepinephrine, and epinephrine were able to favor a Th2 profile because they inhibited macrophage synthesis of IL-12, a major Th1-inducing molecule (65, 113). Similarly, corticosteroid-treated monocytes lose their capacity of inducing the production of IFN-γ by $CD4^+$ T lymphocytes, whereas stimulating their secretion of IL-4 (114). In addition, stress caused by electric shocks raised plasma corticosterone levels and lowered macrophage antitumor activity, favoring the growth of Ehrlich ascites tumor in a mouse model (95). Other forms of stress induction, such as restraining or corticosterone injection, inhibited the production of TNF-α and reactive nitrogen species by macrophages, which thereby increased susceptibility to *Mycobacterium avium* infection (115).

As part of the HPA axis, there is also an acetylcholine-based suppressive neuroinflammatory-macrophage communication that is centered on the stimulation of the vagus nerve by microorganisms or cytokines (116). Anatomically, these paired nerves gather long-range afferents that convey systemic behavior-changing signals into the brain, and efferents from the medulla oblongata that innervate the heart and numerous visceral organs. In organs such as the liver, pancreas, and the gastrointestinal tract, these nervous fibers can exert relevant control of metabolism and tissue homeostasis (116–118). The release of acetylcholine from synaptic nerve endings targets both muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR, respectively), whose subunits are differentially expressed by monocytes and macrophages depending on species, maturation stage, tissue, and degree of cell activation (119–124). Interaction of the vagus nerve with tissue-resident macrophages is not always direct; in the gut, there is the participation of intervening myenteric neurons scattered around the muscularis layer, whereas the spleen shows no evidence of vagal innervation (125).

Stimulation with acetylcholine inhibits mortality associated with the LPS-induced production of macrophage pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, whereas injuries of the vagus nerve may culminate in uncontrolled inflammatory responses (126). In BALB/c mice, vagotomy led to Kupffer cell-dependent fulminant hepatitis upon intraperitoneal co-injection of LPS and D-galactosamine, an effect that was either reduced by nicotine or exacerbated by α-bungarotoxin, a selective antagonist of the nAChR α7 subunit (127). In agreement with these observations, agonist binding to the nAChR α7 subunit induced the Jak2/STAT3 pathway to suppress resident peritoneal macrophage activation and inflammation (128). Actually, each of these signaling components may have clinical standing to ameliorate the inflammatory output. Notwithstanding, electrical stimulation of the vagus nerve through non-invasive devices is a likely alternative to reduce the numbers of drug-based interventions, as experimentally demonstrated in inflammatory bowel disease, kidney ischemia-reperfusion injury, and rheumatoid arthritis

(129–131). Thus, the intricate neuroendocrine regulatory mechanisms of macrophages pose a challenge, but support a role for hormone and neurotransmitter receptors as amenable targets for the development of effective therapeutic strategies based on regulating monocyte and macrophage differentiation, migration, polarization and activation, phagocytosis, or antigen presentation.

TISSUE-SPECIFIC DIFFERENCES IN MACROPHAGE REGULATION BY STRESS MEDIATORS

Glucocorticoids effects on macrophages generally influence the resolution phase of inflammation. Interestingly, their actions can be regulated locally by cell-specific particularities, such as receptor availability and metabolism. One of these mechanisms includes the enzymes 11β-hydroxysteroid dehydrogenases type 1 (11β-HSD1) and type 2 (11β-HSD2) that act upstream of the GR receptor and are ultimately responsible for glucocorticoid metabolism. In general, they play opposing roles in a tissue-dependent context. Whereas 11β-HSD1 converts inactive cortisone to active cortisol in the vasculature, adipose tissue, muscle, liver, and brain, the 11β-HSD2 enzyme inactivates cortisol to cortisone in the kidneys and colon. Thereby, glucocorticoid levels can be controlled independently from the systemic axis [reviewed by Chapman et al. (132)].

Macrophages also differentially express these enzymes. In particular, the expression of 11β-HSD2 is low, whereas the levels of 11β-HSD1 are highest in anti-inflammatory macrophages and high in pro-inflammatory cells when compared to resting macrophages (133, 134). This occurs because the cytokines IL-4 or IL-13 are able to upregulate 11β-HSD1 activity, whereas IFN-γ plays a suppressive role. A higher expression of 11β-HSD1 is also found as monocytes differentiate to anti-inflammatory macrophages. Incubation with LPS produces no alteration on the expression of 11β-HSD1 in monocytes, but it can increase enzyme levels in pro-inflammatory macrophages (133). In microglia, LPS does induce an upregulation of 11β-HSD1 expression, but without an apparent change in protein abundance (135). Likewise, the same stressor may also initiate opposing responses in macrophages, which may result in confusion or difficulty of interpretation. For instance, acute cold stress induces a reduction in the phagocytic activity of resting macrophages that is mediated by corticosterone, but an increase in phagocytosis by activated cells that depends on catecholamines (136). On the other hand, chronic cold stress promotes an anti-inflammatory phenotype that correlates with increased expression of 11β-HSD1 (137). In the adipose tissue, these anti-inflammatory macrophages secrete catecholamines to induce thermogenic gene expression in brown adipose tissue and lipolysis in white adipose tissue (111). In turn, neuron-produced norepinephrine activates tissue-protective programs by muscularis macrophages in the intestine, whereas lamina propria macrophages exhibit pro-inflammatory characteristics (138). Those muscularis macrophages respond similarly to norepinephrine as the microglia in the CNS (139, 140).

Together, these observations point out that tissue-specific macrophages have the ability to respond differently to the same neuroendocrine stimulus such as glucocorticoids, and it suggests the existence of an intricate tissue-dependent network of regulation on macrophage function. A pathological condition can, however, modify macrophage response to neuroendocrine mediators. Similar to alveolar macrophages in acute respiratory distress syndrome, synovial macrophages in osteoarthritis or rheumatoid arthritis present high levels of 11β-HSD2, which may contribute to glucocorticoid resistance and the persistence of chronic inflammation (141–143).

CLINICAL RELEVANCE OF TARGETING MACROPHAGES WITH NEUROENDOCRINE SIGNALS

Both neurons and immune cells are able to sense and respond to exogenous and endogenous challenges, being involved in governing critical homeostatic pathways. It is a remarkable feature the existence of a network allowing these cells to interact with each other *via* cell–cell communication or *via* their main soluble signaling molecules, the neurotransmitters, and cytokines. Considering that these bidirectional communications may be disturbed in immunopathological and neurological diseases, the better understanding of such an intricate body of interactions may help to both unveil new mechanisms of diseases and the search for new therapies. An important homeostatic arm to counteract an inflammatory state is driven by the nervous system *via* triggering the HPA axis and the consequent release of glucocorticoids, and also by activating the sympathetic nervous system to secrete catecholamines. As target cells of these factors, macrophages can be turned to an anti-inflammatory state as glucocorticoids are regarded by their immunosuppressive function and the catecholamines can induce IL-10 macrophage secretion (**Figure 3**) (65, 144). Additionally, macrophages seem to be regulated by the efferent vagus nerve *via* their nicotinic acetylcholine receptors (145). Therefore, aiming at controlling undesired effects of tissue inflammation, one can envisage that interfering on brain-to-macrophage signaling might be an effective strategy to induce regulatory therapies for inflammatory diseases.

In a second hand, the differentiation program of monocyte/macrophage lineages, and their functional activities, following the differentially acquired polarization status, show a more complex pathway for exploring therapeutically the macrophage to brain signaling. Nonetheless, several studies have demonstrated the critical role of monocyte/macrophage recruitment, activation and polarization in tissue injury and in the outcome of disease progression. Indeed, a change in macrophage function is critical at the distinct phases necessary for the restoration of tissue homeostasis. During the regeneration of skeletal muscle, for example, pro-inflammatory monocyte-derived macrophages induce the proliferation and migration of progenitor myoblasts at the injury site (146, 147). The phagocytosis of dying cells then induces a switch of pro-inflammatory macrophages to an anti-inflammatory phenotype that stimulates myoblast fusion and both the repair of damaged muscle fibers and the formation of new ones (148, 149).

The concerted activity of both pro- and anti-inflammatory subsets restores the contractile machinery of muscle fibers and repair the underneath fiber basement membrane. As a proof-of-concept for the delivery of macrophages in clinical application, the coinjection of human myoblasts with pro-inflammatory human macrophages into cryodamaged tibialis anterior muscle of *Rag2*$^{-/-}\gamma C^{-/-}$ immunodeficient mice increased muscle cell proliferation and migration, whereas inflammatory cells transited to a resolutive phenotype that supported muscle differentiation through the production of TGF-β after 5 days (146). On the other hand, blocking the anti-inflammatory tumor-promoting activity of tumor-associated macrophages has been reported as an encouraging antitumor therapy (150–152). Interestingly, this can be achieved by the administration of M(LPS + IFN-γ) macrophages in the affected area, which then recruit endogenous macrophages and instruct them into Ly6C^{lo}CD11b^{hi}F4/80^{+} restorative cells (153).

As for the CNS, one should take into account the broad presence of microglia, the CNS-specific resident macrophages. In this context, as the microglia faces potentially harmful invading entities (e.g., pathogens or tumors), these immunosurveillance cells become activated, triggering a protective inflammatory response. However, dysregulation of this neuroinflammation state might result in tissue damage and neurodegeneration, which show microglial activation as a central pathogenic hallmark (154). This concept points out that approaching microenvironmental polarization in the CNS should be well balanced. Nonetheless, the vast body of fundamental data and clinical studies also indicate that targeting macrophage/microglia activation and polarization should be pursued as a potential therapy for neuroinflammatory diseases. In fact, some clinical and experimental therapeutic approaches for neuroinflammatory conditions are known to induce an anti-inflammatory microenvironment in the CNS with increased expression of type-2 cytokines. Such polarization seems to result in both immunosuppressive and regenerative effects, with the production of anti-inflammatory cytokines and neurotrophic factors, including TGFβ, IL-10, IGF-1, and BDNF (155). Thus, experimental studies aiming at the generation of a Th2 and anti-inflammatory microenvironment by carrying IL-4 expression through viral vectors to the CNS in a murine model of multiple sclerosis, resulted in significant reduction in neuroinflammation and neurodegeneration [reviewed in Ref. (155)]. Similarly, the therapy with synthetic polypeptides that resemble myelin basic protein, known as glatiramer acetate, was reported to induce type-2 cytokines and BDNF production by immune cells, and play an immunomodulatory effect on the relapsing form of multiple sclerosis (156). Accordingly, the presence of anti-inflammatory (or immunosuppressive) macrophages reduces pro-inflammatory components and might offer support for neuronal survival. In fact, enhanced expression of BDNF has been described in activated macrophages and microglia following brain injury (157, 158). Also, a switch to an anti-inflammatory Arg-1^{+}CD68^{+} phenotype in microglia and peripherally derived macrophages was shown to correlate with remyelination and to support oligodendrocyte differentiation in a murine model of CNS demyelination (159).

The complex nature of macrophage polarization also shows the potential role of CD14^{+}CD16^{+}CD163^{+}CD204^{+}CD206^{+} CD209^{-} macrophages in the resolution of an inflammatory state. Anti-inflammatory M(M-CSF) or M(GC) macrophages highly express the MerTk receptor for apoptotic cells (55), a feature that points out this phenotype as a further target in neuroinflammatory conditions as the neuronal protection and survival requires an efficient clearance of apoptotic cells and debris. Engulfment of apoptotic cells by M(M-CSF + IL-10) macrophages integrates resolution of inflammation with proper tissue repair and the consequent waning of the neurodegeneration process (55).

The immunomodulatory role of the cholesterol-lowering drug atorvastatin on macrophage function has also been explored as a potential anti-neuroinflammatory agent. In a murine model of traumatic brain injury, this drug inhibited microglia/macrophage activation and showed enhanced anti-inflammatory polarization (160). Interestingly, atorvastatin has been reported to downmodulate activation of a blood monocyte subset that seems to be involved in HIV-1-associated neurocognitive disorders (161). Since this compound can accumulate in the CNS, its effects on both recruited inflammatory monocytes and microglia have been a matter of clinical investigation (https://clinicaltrials.gov/, ID: NCT01600170). Altogether, these data gather some interesting concepts derived from basic studies regarding the regulation of macrophage activities on homeostatic and pathological conditions. More importantly, the evidence of macrophage interplay with components of the nervous system and their functional role in neuroinflammatory conditions mounts progressively, hopefully enticing new clinical studies on more efficient treatment of cancer, inflammatory and neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

AJ conceived the review subject, analyzed transcriptome data, and wrote the paper. VC-d-A and JT wrote the paper. WS conceived the review subject and wrote the paper. DB-H and IR conceived the review subject, coordinated the work, and wrote the paper. Artwork by AJ and IR.

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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.2018.01440/full#supplementary-material.

FIGURE S1 | Monocytes and macrophages express many neuroendocrine-related genes. Gene expression of selected neurotransmitters and hormone receptors (columns) in distinct monocyte and macrophage populations (rows) distributed throughout the body. The ImmGen dataset was plotted as heatmap with hierarchical clustering as described by Beyer et al. (162), using a coefficient of variation of 20%. Levels of expression are represented by colors in which red, white, and blue indicate high, intermediate, and low intensities, respectively.

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Into the Moment: Does Mindfulness Affect Biological Pathways in Multiple Sclerosis?

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Mindfulness was introduced in the Western world by Jon Kabat-Zinn in 1979. He defined it as "awareness that arises through paying attention, on purpose, in the present moment, non-judgmentally." Since then, research on mindfulness-based interventions (MBIs) has increased exponentially both in health and disease, including in patients with neurodegenerative diseases such as dementia and Parkinson's disease. Research on the effect of mindfulness and multiple sclerosis (MS) only recently gained interest. Several studies completed since 2010 provided evidence that mindfulness improves quality of life (QoL), depression and fatigue in MS patients. In addition to patient-reported outcome measures, potential effects on cognitive function have been investigated only to a very limited extent. However, research on laboratory biomarkers and neuroimaging, capable to deliver proof-of-concept of this behavioral treatment in MS, is mainly lacking. In this perspective, we illustrate possible neurobiological mechanisms, including the tripartite interaction between the brain, the immune system and neuroendocrine regulation, through which this treatment might affect multiple sclerosis symptoms. We propose to (1) include immunological and/or neuroimaging biomarkers as standard outcome measures in future research dedicated to mindfulness and MS to help explain the clinical improvements seen in fatigue and depression; (2) to investigate effects on enhancing cognitive reserve and cognitive function; and (3) to investigate the effects of mindfulness on the disease course in MS.

Keywords: multiple sclerosis, mindfulness, fatigue, depression, cognitive function, immune system, stress, MRI

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INTRODUCTION

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic neuroinflammatory and neurodegenerative disease with an unpredictable course that affects more than 2.5 million people worldwide. It is the main cause of nontraumatic disability in young adults in many countries (Browne et al., 2014). The disease course can be relapsing or progressive (Lublin et al., 2014). Varying visible (mainly physical) and unvisible or hidden symptoms occur in MS. The hidden symptoms, such as fatigue, depression, psychological distress, and cognitive dysfunction pose a significant burden on the patient's quality of life (QoL) and workforce participation. There is a high and unmet need for treatments able to tackle these hidden symptoms (Lysandropoulos and Havrdova, 2015).

These invisible symptoms are highly prevalent in MS patients. Cognitive dysfunction occurs in 40–70%, depression in 30–40% while fatigue can be present in up to 85–95% of patients (Feinstein et al., 2014; Rocca et al., 2015; Patejdl et al., 2016). Cognitive dysfunction can start early in the disease course, even at the stage of clinically isolated syndrome (CIS) (Rocca et al., 2015; Benedict et al., 2017). The most frequently affected domains are information processing speed and episodic memory (Van Schependom et al., 2015; Hämäläinen and Rosti-Otajarvi, 2016; Köhler et al., 2017). Although self-reported cognitive dysfunction in patients with MS is only moderately correlated with objective cognitive impairment, it has a negative impact on QoL and is often related to mood and fatigue (Rosti-Otajärvi et al., 2014; Strober et al., 2016). It is well known that depression has adverse effects on cognitive functioning, including working memory, executive functioning, and information processing speed (Feinstein et al., 2014).

Neurobiology of Hidden Symptoms in MS

The proposed underlying biological mechanisms for these hidden symptoms show some common and overlapping features. Neuroinflammation in the peripheral circulation and the central nervous system (CNS) has a role in cognitive dysfunction (Berger, 2016), fatigue (Hanken et al., 2014; Patejdl et al., 2016), but also in anxiety and depression in MS (Feinstein et al., 2014; Patejdl et al., 2016; Rossi et al., 2017). Interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) in the cerebrospinal fluid (CSF) have been shown to correlate with mood disorders (Rossi et al., 2017), while serum interleukin-6 (IL-6) and increases in interferon-gamma (IFN-γ) and TNF-α (using whole-blood-cytokine-stimulation) correlate with fatigue (Patejdl et al., 2016). Growth factors may also play a role in the neurobiology of the invisible symptoms. A decrease in brain derived neurotrophic factor (BDNF) in early MS patients has been demonstrated and was associated with cognitive impairment in one recent study (Prokopova et al., 2017). It is well known that BDNF is a biomarker for depression, although this correlation remains to be shown in MS patients, whose immune cells secrete lower than normal BDNF (Azoulay et al., 2008; Kishi et al., 2017). After physical training in MS patients, an increase in BDNF can be paralleled by improved fatigue (Ozkul et al., 2018). Therapeutic strategies to decrease neuroinflammation and to increase BDNF might thus be helpful in alleviating hidden symptoms.

To date, serum biomarkers to monitor disease activity in MS don't exist, although neurofilament-light levels may hold promise in this regard (Novakova et al., 2017). Brain Magnetic Resonance Imaging (MRI) is currently regarded as a golden standard in MS to follow subclinical disease activity, consisting of gadolinium enhancing T1 lesions and new or enlarging T2 hyperintense lesions. MRI measurements of whole brain volume, and more specifically gray matter volume correlate well with disease progression, disability, and cognitive dysfunction (Rocca et al., 2015; Wattjes et al., 2015). On the other hand, MRI T2 lesion load is only weakly correlated with hidden symptoms (Feinstein et al., 2014; Rocca et al., 2015; Patejdl et al., 2016). MRI abnormalities may already occur before cognitive symptoms are noticeable (Roosendaal et al., 2010; Rocca et al., 2015). Hippocampal atrophy in MS patients has been correlated with both depression and cognitive dysfunction. This has been linked to increased cortisol levels (Feinstein et al., 2014). Both hippocampal and deep gray matter nuclei atrophy are predictors of cognitive impairment in MS and these measurements might be useful as biomarkers in treatment studies (Damjanovic et al., 2017; Köhler et al., 2017). While the correlation between certain structural MRI measures and cognition and depression shows common features, this is less clear for fatigue (Patejdl et al., 2016). MRI in fatigued MS patients correlates better with lesions and atrophy in prefrontal, thalamic and temporal structures (Patejdl et al., 2016). A decrease in the cortical thickness in the right middle temporal pole has been described in MS patients who are both fatigued and depressed (Hanken et al., 2016). To study brain plasticity, functional MRI (fMRI) is of interest (Enzinger et al., 2016). fMRI has led to the hypothesis that fatigue is a consequence of increased brain activation due to cortical reorganization, loss of connectivity and delayed processing, with important roles for the thalamus and frontoparietal cortex (Patejdl et al., 2016). Decreased connectivity in the anterior regions of the brain also plays a role in cognitive dysfunction and depression (Feinstein et al., 2014; Rocca et al., 2015).

To summarize, a proinflammatory cytokine profile and lower than normal BDNF levels seem to play a role in MS related fatigue, mood disorders, and cognitive function. Hippocampal and deep gray matter atrophy are correlated with cognitive dysfunction and depression, while fatigue is related to structural abnormalities in frontal, temporal but also deep gray matter regions.

Treatment of Hidden Symptoms

The current treatment approach of cognitive dysfunction is to try to prevent cognitive impairment by using immunomodulatory drugs early in the disease course and when cognitive symptoms and/or impairment are present to resort to cognitive rehabilitation (Miller et al., 2017). Interestingly, perceived cognitive deficits can also improve after a neuropsychological rehabilitation program (Rosti-Otajärvi et al., 2013). Another preventive strategy is based on enhancing brain and cognitive reserve via a so-called brain-healthy lifestyle, including physical exercise (Crescentini et al., 2014; Sumowski et al., 2014; Sumowski, 2015; Sandroff et al., 2016; Kobelt and Giovannoni, 2017). Several studies have shown evidence for some beneficial effects of physical training on cognitive function, MRI measurements, neurotrophins, and/or immune markers in MS patients (Bansi et al., 2013; Leavitt et al., 2014; Kierkegaard et al., 2016; Wens et al., 2016; Feys et al., 2017; Kjølhede et al., 2017; Sandroff et al., 2017; Zimmer et al., 2017). A recent Cochrane review concluded that there is some evidence in favor of effectiveness of memory rehabilitation on memory function and on QoL. (Prosperini et al., 2015; das Nair et al., 2016; Hämäläinen and Rosti-Otajarvi, 2016). Non-pharmaceutical interventions to improve MS-related fatigue such as fatigue management and enhancing physical activity may be equally effective as pharmaceutical treatment such as amantadine (Brenner and Piehl, 2016; Tur, 2016). The treatment

of depression in MS generally follows the guidelines for the general population, using pharmacological treatment with SSRI's (selective serotonin reuptake inhibitors) or CBT (cognitive behavioral therapy) (Brenner and Piehl, 2016). Despite these therapeutic approaches, hidden symptoms remain difficult to treat in clinical practice.

In conclusion, there are important relationships between subjective and objective cognitive dysfunction, depression, fatigue and QoL in MS and some of these symptoms show correlation with neuroimaging markers or immunological markers. Furthermore, there remains an unmet need for hypothesis-driven effective interventions that target these interrelated MS symptoms altogether and that have the ability to enhance brain and cognitive reserve.

MINDFULNESS

Mindfulness was introduced in the Western world in 1979 by Jon Kabat-Zinn who defined it as "awareness that arises through paying attention, on purpose, in the present moment, non-judgmentally" (Kabat-Zinn, 1982). This meditation technique is based on traditional Buddhist meditation practices. The main goal is to enhance self-regulation via amplification of attentional control, improving emotional regulation and changing self-awareness (Tang et al., 2015). While the classic program, as developed by Jon Kabat-Zinn, finds its roots in mindfulness meditation and is named Mindfulness-Based Stress Reduction or MBSR, adapted programs are referred to as Mindfulness-Based Interventions or MBIs (Shonin et al., 2013) or Mindfulness Based Programs or MBPs (Crane et al., 2017). Since its introduction (Kabat-Zinn, 1982), numerous studies have been conducted on the immunological and neuroimaging effects of mindfulness in both health and disease, albeit not in MS. However, the quality of these studies has been highly variable due to inherent limitations of the intervention and control conditions (prone to bias due to self-report measures, short-term studies, small sample size, lacking active control groups, heterogeneity) (Goldberg et al., 2017; Kabat-Zinn, 2017).

As in MS, fatigue, cognitive symptoms and depression are often interrelated, mindfulness might be a way of dealing with all these problems at the same time (Senders et al., 2014). Here, we will review the beneficial effects of mindfulness that have been demonstrated in MS, describe effects of mindfulness on the immune system and MRI, explain the relationship between hidden symptoms in MS, immunology and MRI, elaborate on potential effects of mindfulness on cognitive function and disease course in MS, hypothesize on underlying neurobiological mechanisms, and finally suggest avenues for further research including laboratory biomarkers and neuroimaging.

Clinical Effects

To date, the feasibility of MBIs as an intervention for MS patients has been demonstrated in several studies (see **Table 1**). The studies comprised different types of MS patients, and incorporated a variety of MBIs, including even telemedicine, Skype, and/or online training (Grossman et al., 2010; Senders et al., 2014; Bogosian et al., 2015; Kolahkaj and Zargar, 2015; Frontario et al., 2016; Nejati et al., 2016; Blankespoor et al., 2017; Gilbertson and Klatt, 2017; Hoogerwerf et al., 2017; Simpson et al., 2017). A MBI has been shown to positively influence QoL, depression, anxiety and fatigue in MS patients in an RCT (intervention vs. waiting list control group) with a sample size of 150 patients and rigorous study design (pre- and post-intervention and 6 months follow-up) (Grossman et al., 2010). These beneficial effects have been confirmed in other RCTs that used an active control group (psychoeducational group) (Carletto et al., 2017; Cavalera et al., 2018). Moreover, sleep problems and illness perception improved to a greater extent in the intervention group than in the control group (Carletto et al., 2017; Cavalera et al., 2018). While the beneficial effects lasted for 6 months after conventional MBI (Carletto et al., 2017), the effects could not be maintained for 6 months with online mindfulness training (Cavalera et al., 2018).

There is some evidence to support positive effects of MBIs on cognition, including selective and executive attention, working memory, executive functions and cognitive flexibility, in healthy subjects (extensively reviewed by Chiesa et al., 2011). The effect of MBIs on cognition in MS patients has not been studied extensively, albeit its effect was explored in a number of pilot studies with variable results (Frontario et al., 2016; Blankespoor et al., 2017; Simpson et al., 2017). The first assessed the effects of a telemedicine mindfulness intervention in patients with MS in a randomized-controlled way and found that the Symbol Digit Modalities Test (SDMT) improved more in MS patients who underwent the intervention as compared to the control group who only had a one-time instruction (Frontario et al., 2016). In another pilot RCT, self-reported prospective memory had improved at 3 months post-intervention. However, the authors did not include objective cognitive measurements in this study (Simpson et al., 2017). More recently, a pilot study explored the effects of mindfulness on psychological functioning, QoL and cognitive function in a convenience sample of Dutch MS patients. In agreement with previous findings, significant improvements were found in depressive symptoms, QoL and fatigue. Moreover, improvement on a visual spatial processing test was reported after the intervention (Blankespoor et al., 2017). Also, Grossman et al. (2010) studied cognitive function, although this was not a primary nor key secondary outcome. The treating neurologist performed cognitive testing with the Multiple Sclerosis Inventory of Cognition (MUSIC), but results were not reported as there were no differences in these measures pre- and post-intervention nor at 6 months follow-up (*personal communication, P. Grossman*).

Although a more harmonized way of patient monitoring should be strived for, the above-mentioned observations underscore that MBIs should be considered as a valuable behavioral treatment option for MS patients capable to improve their mental well-being.

Biological Outcome Measures

While a plethora of biological outcome measures are influenced by physical interventions in MS, including TNF-α, BDNF, IL-6, matrix metalloproteinases (MMP), soluble receptor of IL-6 (sIL-6R), nerve growth factor (NGF), and/or brain MRI measures

TABLE 1 | Mindfulness and MS in clinical studies.

References	Study design	Sample size	Key inclusion criteria	Intervention	Control	Post-intervention follow-up	Primary outcome	Secondary outcome	Results
Cavalera et al., 2018	RCT	139	RRMS and SPMS	8-week online course via Skype	Psychoeducational group	PI, 6 M	QoL	Anxiety and depression, sleep, fatigue	+ QoL, anxiety, depression, sleep at PI (– at 6 M)
Carletto et al., 2017	RCT	90	MS, depressive symptoms	Body-affective mindfulness	Psychoeducational group	PI, 6 M	Depression	Fatigue, perceived stress, illness perception	+ Depression, perceived stress, illness perception, QoL – Fatigue
Simpson et al., 2017	RCT	50	MS	MBSR	Waiting list	PI, 3 M	Feasibility, perceived stress, QoL	QoL, self-compassion, common MS symptoms	+ Perceived stress, anxiety, depression, self-compassion, positive affect at PI + Mindfulness, positive affect, self-compassion, anxiety, prospective memory at 3 M Feasible
Hoogerwerf et al., 2017	Non-randomized controlled	59	RRMS and SPMS, severe fatigue	MBCT	Patient is his/her own control	PI, 3 M	Fatigue	Anxiety and depression, coping, sleep, mindfulness	Feasible + Fatigue, anxiety, depression
Blankespoor et al., 2017	Open-label, pilot	25	MS	MBSR	No control	PI	Self-report and neuropsychological testing		+ Visual spatial processing, depressive symptoms, QoL, fatigue, mindfulness, self-compassion
Gilbertson and Klatt, 2017	Open-label, feasibility	20	MS	Mindfulness in motion	No control	PI	Feasibility	Fatigue, depression, anxiety, QoL	Feasible+ depression, anxiety, fatigue
Nejati et al., 2016	Controlled trial	24	MS	MBSR	Usual care?	PI	QoL, fatigue severity		+ QoL and fatigue severity
Frontario et al., 2016	Pilot RCT	30	MS	MBI based on MBSR, teleconference	One-time introduction to MBI	PI	SDMT and PASAT	Depression, fatigue	+ SDMT, PASAT, depression, and fatigue
Kolahkaj and Zargar, 2015	RCT	48	MS, females only	MBSR	Usual care	PI, 1 M	Anxiety, depression, stress		+ Anxiety, depression, stress
Bogosian et al., 2015	Pilot RCT	40	SPMS and PPMS	Mindfulness based on MBCT via Skype	Waiting list	PI, 3 M	Distress	Depression and anxiety, MS impact, pain, fatigue, QoL	+ Pain (only at 3 M), anxiety, depression, MS impact psychological
Grossman et al., 2010	RCT	150	RRMS and SPMS	MBI based on MBSR	Waiting list	PI, 6 M	QoL, depression, fatigue	Anxiety, perceived personal goal attainment, self-reported homework	+ QoL, well-being

Overview of clinical studies investigating feasibility and/or efficacy of MBIs in MS since 2010. Study design, sample size, intervention, and control condition, follow-up, primary, and secondary outcomes and results are presented. RCT, randomized controlled trial; MS, all types of multiple sclerosis; RRMS, relapsing remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; MBSR, mindfulness based stress reduction; MBCT, mindfulness based cognitive therapy; MBI, mindfulness based intervention; PI, post-intervention; 1 M, 1 month; 3 M, 3 months; 6 M, 6 months; +, positive effect; –, no effect.

(functional, gray matter) (Bansi et al., 2013; Leavitt et al., 2014; Deckx et al., 2015, 2016; Kierkegaard et al., 2016; Wens et al., 2016; Feys et al., 2017; Sandroff et al., 2017; Zimmer et al., 2017), research on the effects of mindfulness on biomarkers in MS remains elusive. Nonetheless, findings of MBIs in other study subjects, which will be outlined below, underscore the need to take into account soluble factors in future studies in MS.

Mindfulness and Immunology

To date, there is only limited evidence that mindfulness meditation modulates the immune system. Mostly reported is a reduction in proinflammatory cytokine levels. Indeed, in a review of 20 RCTs of which 50% included an active control group and 50% a waiting list control group, a decrease in IL-6, TNF-α, and CRP as well as an increase in IL-8, IL-10, and IFN-γ was noted (Black and Slavich, 2016; Sanada et al., 2017). While in healthy subjects, MBIs had no effect on cytokine levels, except for an increase of insulin-like growth factor 1 (IGF-1) (Sanada et al., 2017), the effects were more pronounced in cancer patients, albeit with contradictory results including changes in the concentration of IFN-γ and IL-4, a decrease in TNF-α and IL-10, and no differences in the level of IL-6 (Sanada et al., 2017). One of the issues is that current markers have been measured in the peripheral blood rather than in the CNS and as such don't truly reflect the underlying biological process.

Furthermore, MBIs might have an impact on the expression of genes that are induced by stress. Several studies consistently showed a downregulation of the NFkB pathway, while in chronic stress this pathway is upregulated, leading to increased inflammation (Creswell et al., 2012; Kaliman et al., 2014; Buric et al., 2017). Hence, MBIs may lead to a reduction in NFkB-mediated inflammation. Interestingly, this pathway is also implicated in MS pathology and one of the current DMTs, dimethyl fumarate, modulates this pathway (Mc Guire et al., 2013; Miljković et al., 2015).

While one might expect a reduction in proinflammatory cytokines in serum and/or CSF as well as changes in gene expression of stress-induced genes in MS patients based on these data, this hypothesis remains to be investigated.

Mindfulness and MRI

A meta-analysis by Fox et al. (2014) reviewed 21 neuroimaging studies including approximately 300 meditation practitioners and found 8 brain regions that were most consistently changed in meditators. These were brain areas related to meta-awareness (frontopolar cortex), body awareness (sensory cortex and insula), memory (hippocampus), self and emotion regulation (anterior and mid cingulate; orbitofrontal cortex), and intra- and interhemispheric communication (superior longitudinal fasciculus; corpus callosum), all functions that are supposed to be enhanced through meditation. The global effect size was termed as medium (Cohen's $d = 0.46$; $r = 0.19$) (Fox et al., 2014). A more recent review (Gotink et al., 2016) on brain changes induced by mindfulness included 30 studies, concerning healthy subjects and patients. In both healthy as well as anxious and stressed participants, the activity, connectivity and volume of the prefrontal cortex, the cingulate cortex, the insula, and the hippocampus were increased. Specific changes in the amygdala included a decrease in functional activity, improvement in functional connectivity with the prefrontal cortex, and earlier deactivation after exposure to emotional stimuli. These results show similarities with the brain changes seen in meditation practitioners.

However, due to methodological limitations of the included studies and a concern for publication bias (Coronado-Montoya et al., 2016), more research is needed to confirm these findings. Of interest, brain regions that are influenced by MBIs are involved in hidden MS symptoms, as previously described. Adding structural and/or functional neuroimaging in research on the effects of MBIs in MS may improve the knowledge in this domain.

HOW MINDFULNESS MIGHT AFFECT BIOLOGICAL PATHWAYS AND HIDDEN SYMPTOMS IN MS

To date, brain MRI nor biological samples have been included as outcome measures in studies on MBIs and MS to explain underlying neurobiological mechanisms of the positive outcomes (Grossman et al., 2010). Cognitive outcomes remain underexplored or underreported. However, some positive effects of MBIs on cognitive measures in Alzheimer's disease and hippocampal neuroplasticity in healthy people and Parkinson's disease have been observed in small studies (Pickut et al., 2013; Larouche et al., 2015; Quintana-Hernández et al., 2016). As described before, MBIs may induce changes in the brain that are of importance to emotional regulation and that might be relevant to enhance cognitive reserve or improve cognitive function in MS patients. To date, there is some preliminary evidence for a neuroprotective role from MBIs, potentially regulated by decreasing glucocorticoid levels and increasing BDNF (Tang et al., 2015). Thus, MBIs hold the potential to improve cognition in a diseased brain via structural brain changes that can be demonstrated directly post-intervention, as has been shown in a study on effects of a MBI in Parkinson's disease (Pickut et al., 2013).

Another way in which MBIs might improve the outcome for MS patients is by stress reduction, thereby influencing the disease course. Stress activates the hypothalamic-pituitary-adrenal axis (HPA-axis), to prepare the body for the "fight or flight" reaction. Although the role of stress in the risk of developing MS is debated, the literature on the association of relapses and stress is more consistent, pointing toward a higher risk of relapses after stressful life events (Artemiadis et al., 2011). Measuring stress however, is not easy and often relies on self-reporting. Biological outcome measures such as measuring autonomic nervous system response are another option (Briones-Buixassa et al., 2015). Stress reduction via a stress management program (SMT) can reduce brain inflammation in MS as measured by a decreased number of enhancing MRI lesions as compared to a control group in an RCT (Mohr et al., 2012). Moreover, MBIs are designed to reduce stress and have demonstrated an impact on several physiological markers of stress in different patient groups (Pascoe et al., 2017). Acute stress may disrupt blood-brain-barrier

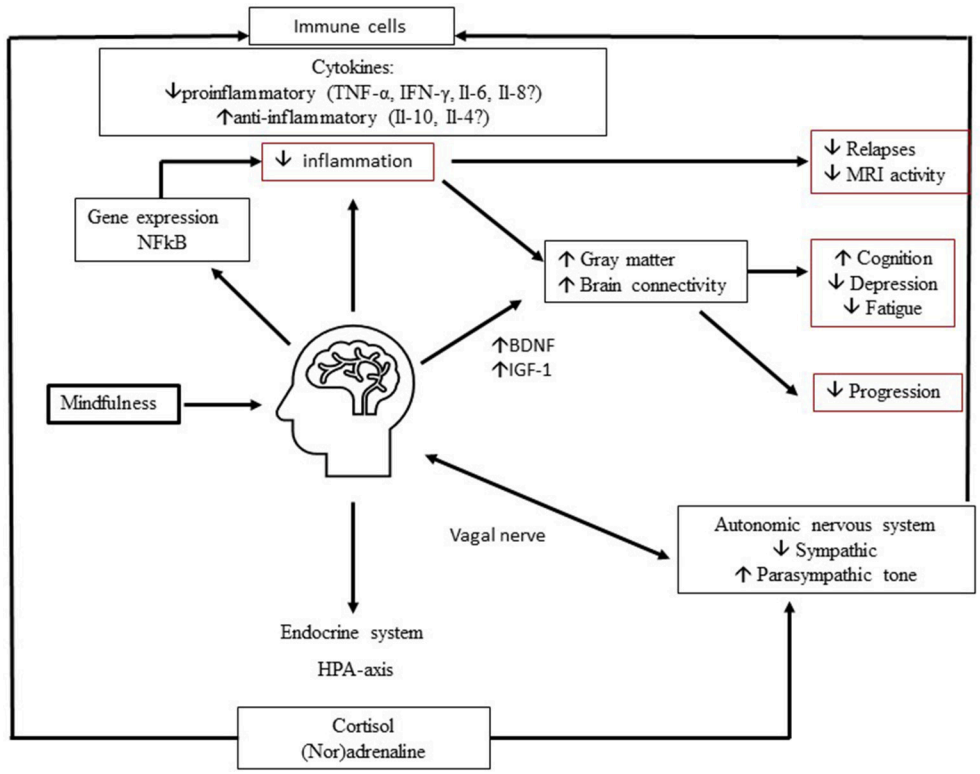

FIGURE 1 | Proposed neurobiological mechanisms of mindfulness in MS. Mindfulness improves depression and fatigue and may improve cognitive function through complex multidirectional neurobiological mechanisms. Involvement of (1) endocrine system, (2) automatic nervous system, (3) growth factors, (4) gene expression may lead to decrease in inflammation and increase in Brain Derived Neurotrophic Factor (BDNF). In turn, this may lead to preserved and improved connectivity and gray matter volume. These mechanisms ultimately improve MS related invisible symptoms and possibly the disease course.

(BBB) permeability via corticotropine releasing hormone (CRH), neurotensin, and activate mast cells and microglia releasing pro-inflammatory cytokines and thereby attracting myelin-reactive T cells that cause MS relapses. Chronic stress, however, may lead to glucocorticoid resistance of immune cells (Gold and Heesen, 2006; Heesen et al., 2007a,b; Deckx et al., 2013; Karagkouni et al., 2013). In MS, hyperactivity of the HPA-axis has also been correlated with cognitive impairment (Heesen et al., 2002, 2010; Gold et al., 2005). A systematic review and meta-analysis studying physiological markers of stress following all types of meditation in different study populations showed that meditation reduced the serum levels of cortisol, C-reactive protein (CRP), triglycerides and TNF-α, as well as blood pressure and heart rate (Pascoe et al., 2017).

In **Figure 1** we show a hypothetical framework to explain neurobiological effects of MBIs in MS. Depression and fatigue improve via a decrease in proinflammatory cytokines and an increase in anti-inflammatory cytokines, mediated via the endocrine system and autonomic nervous system. Also, changes in gene expression may lead to a decrease in inflammation and improvement in symptoms and maybe the disease course. Moreover, through increases in nerve growth factors like BDNF, brain connectivity and gray matter volume in certain brain regions may increase and lead to a decrease in depression, an improvement in fatigue and possibly improve cognitive function and slow down disease progression.

FUTURE DIRECTIONS AND CONCLUSION

In the past decade, the interest in neurobiological effects of behavioral interventions has rapidly increased. In MS, MBIs have demonstrated a positive impact on several hidden symptoms but also hold the potential to influence the disease course itself via various multidirectional brain- to -immune communications, including neuroendocrine, autonomic nervous system, immune and gene expression pathways leading to a decrease in inflammation and enhancing neuroplasticity (see **Figure 1**). MBIs may cause neuroplasticity in structures and functions of specific brain regions, important in attention, memory, emotional regulation, and self-awareness (Fox et al., 2014). In MS, some of these regions are implicated in pathophysiology of depression and fatigue, but also cognitive function. As therapeutic options to treat cognitive impairment in MS are limited, it is an interesting option to investigate effects of MBIs on enhancing cognitive reserve and cognitive function. Although current findings on research on MBIs and biomarkers must be interpreted with caution due to several limitations of the studies

(prone to bias due to self-report measures, short-term studies, small sample size, lacking active control groups, heterogeneity), there are consistent effects seen in MRI and several soluble markers that require replication but also necessitate further in-depth research in the field of MS. While in research on exercise and MS, the use of biological outcome measures has taken an entry (Bansi et al., 2013; Leavitt et al., 2014; Kierkegaard et al., 2016; Wens et al., 2016; Feys et al., 2017; Sandroff et al., 2017; Zimmer et al., 2017), in studies on behavioral interventions and MS this is still a largely unexplored area. Therefore we propose to include immunological and/or neuroimaging biomarkers as standard outcome measures in future research dedicated to mindfulness and MS to help explain the clinical improvements seen in fatigue and depression. Effects of a MBI on cognitive function (subjective and objective) and biomarkers (MRI, cytokines, BDNF) are being investigated in the exploratory MIND-MS study (performed by the authors). This study involves 20 MS patients that undergo a MBI, in an open label study with 3 evaluation timepoints pre- and post-intervention and at 6 months, including cognitive outcomes, structural MRI and measurement of cytokines and BDNF. An RCT called REMIND-MS is currently ongoing and comparing the effects of cognitive rehabilitation with mindfulness vs. a control condition in the Netherlands, making use of MEG (magneto- encephalography) as secondary outcome measure (Nauta et al., 2017). It remains to be shown in MS patients, whether MBIs can have an impact on neuroinflammation and thus putatively on the disease course. Research on the effects of mindfulness on the disease course in MS, using conventional MRI measures and neurofilament-light levels should be done.

Mindfulness can only become an important adjuvant therapy to the current treatment modalities to improve outcomes in MS patients, when investigated in future well-designed clinical trials that follow the patients long-term, making use of not only clinical but also biological and imaging outcome measures, in doing so providing proof-of-concept of the neurobiological mode of action.

AUTHOR CONTRIBUTIONS

BW concepted and drafted the manuscript; GP, PC, and NC critically revised the manuscript. All authors approved the submitted version of the manuscript.

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