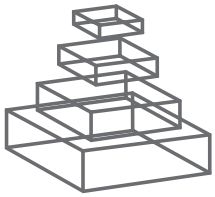
frontiers
RESEARCH TOPICS

BRAIN-IMMUNE INTERACTIONS IN HEALTH AND DISEASE

Topic Editors
Adam Denes and Jaleel A. Miyan

frontiers in NEUROSCIENCE
frontiers in ENDOCRINOLOGY



frontiers

ISSN 1664-8714
ISBN 978-2-88919-514-5
DOI 10.3389/978-2-88919-514-5

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

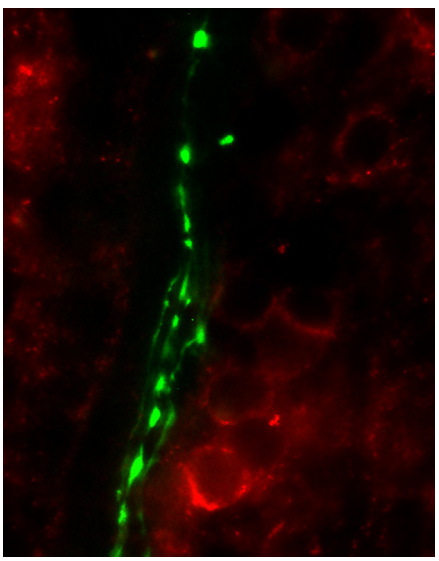
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

BRAIN-IMMUNE INTERACTIONS IN HEALTH AND DISEASE

Topic Editors:
Adam Denes, Institute of Experimental Medicine, Hungary; University of Manchester, UK
Jaleel A. Miyan, University of Manchester, UK



NPY-positive (green) nerve fibers are found in close proximity to myeloid cells (red) in the femoral bone marrow.

Image credit: "Image property of the Denes lab"

Brain-immune interactions are essential to maintain health and their dysfunction contributes to diverse human diseases. Recent data show that haematopoietic processes and immune organs are under central autonomic control. Deficient regulation of inflammatory events contributes to brain diseases, whereas acute or chronic brain injury is linked with the development of systemic inflammatory conditions or immunosuppression. At present, common disorders with high socio-economic burden such as cancer, cardiovascular-, neuroinflammatory- and neurodegenerative diseases, asthma, allergies, autism, psychiatric conditions and sepsis are believed to be influenced, at least in part, by the dysfunction of brain-immune communication. Since the median age of the world's population is increasing rapidly, it is expected that the burden of common non-communicable diseases will further increase, which represents a huge challenge to the health care systems worldwide. Thus, there is an increasing demand to understand and treat complex diseases, many of which are age-related, and this is not possible unless the fine-tuned communication between large systems -such as the nervous and the immune system- is comprehensively understood.

Although it is impossible to cover all areas of relevant research in this field, papers in this eBook give some insight to a few important aspects of brain-immune interactions and their contribution to disease. We hope that this collection could stimulate further relevant research and facilitate discussions to support the understanding of the highly complex interactions between the immune system and the brain in health and disease.

Table of Contents

frontiers in
NEUROSCIENCE

EDITORIAL
published: 03 December 2014
doi: 10.3389/fnins.2014.00382

Brain-immune interactions in health and disease

***Adam Denes*[1,2]* and *Jaleel A. Miyan*[1]**

[1] *Faculty of Life Sciences, University of Manchester, Manchester, UK*
[2] *Laboratory of Molecular Neuroendocrinology, Institute of Experimental Medicine, Budapest, Hungary*
**Correspondence: adam.denes@manchester.ac.uk*

Edited and reviewed by:
Hubert Vaudry, University of Rouen, France

Keywords: brain-immune interactions, neuroinflammation, systemic responses, bi-directional communication, disease

Modern medicine cannot avoid the understanding of the fine-tuned communication between the many, seemingly distinct systems in the body. An excellent example of this is the challenge to understand the bi-directional communication between the brain and the immune system. Brain-immune interactions take place in different organs, involving a wide range of cells and mediators, coordinated through sensory and effector pathways in the central nervous system (Ader et al., 1990; Elenkov et al., 2000; Rivest, 2009). The interactions work in both directions to maintain a healthy state of body and brain in the face of diverse, harmful challenges from foodstuffs, toxins, allergens, infective agents, or injury. Dysfunction and inappropriate regulation of inflammatory or neuronal responses underlie many diseases that have become more prevalent in recent decades, predominantly in developed countries. These countries are also characterized by an increased aging population and profoundly increased cost to healthcare due to age-related brain conditions including dementia and other neurodegenerative diseases. Recent research has established a significant role for the immune system in several brain diseases including multiple sclerosis, tumors, stroke, mental disorders, Alzeimer's, and Parkinson's disease. In turn, mood disorders, stress, autonomic dysfunction, acute, and chronic brain injury have been linked with the development of organ failure, cancer, heart disease, systemic inflammatory conditions, infections, and hematological diseases further implicating dependent interrelationships between the immune system and the brain (Denes et al., 2010; Moreno-Smith et al., 2010; Deretzi et al., 2011; Iadecola and Anrather, 2011; Wraith and Nicholson, 2012; Theoharides et al., 2013; Heneka et al., 2014). Both preclinical and clinical research have contributed significantly to our knowledge about these interactions, yet another major challenge is to translate multiple research findings into clinical benefit.

The papers in this research topic discuss some of the most pressing issues concerning the interactions between the neural and immune systems. Murakami and colleagues present their research findings and their "gateway" theory of how regional neuronal responses can drive the migration of autoreactive T cells across the cerebrovascular endothelium to particular sites of the brain where they contribute to the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (Kamimura et al., 2013). They also show that regional neural stimulation can therapeutically prevent the gating through blood vessels. Geenen et al. discuss how autoimmunity directed against neuroendocrine glands could be due to genetic or acquired problems that affect the presentation of neuroendocrine self-peptides in the thymus (Geenen et al., 2013). This process in the thymus is normally responsible for the clonal deletion of self-reactive T cells and the generation of regulatory T cells. Their findings could thus support the development of novel treatment strategies against type 1 diabetes for example.

In their comprehensive review article, Anrather and colleagues describe how reprogramming of local and systemic immune mechanisms contributes to the induction of cerebral ischemic tolerance, a process that is characterized by protection against the ischemic injury after application of ischemic stress to one tissue or organ (Garcia-Bonilla et al., 2014). Appropriate reprogramming of key immune mechanisms could be used to develop novel stroke therapies including possible prevention of injury through stroke in vulnerable individuals. The research paper by Denes et al. demonstrates that brain injury, anesthesia, and surgical interventions have diverse systemic consequences, including altered leukocyte responses in several organs of the body and rapid mobilization of granulocytes (Denes et al., 2013). This could have important implications for animal models of cerebral ischemia as well as for patients with brain injury or for those undergoing surgeries or exposed to prolonged anesthesia. The review article by Möller and his colleagues focuses on the regulation of the kynurenine pathway by inflammatory mediators and how this contributes to neurodegenerative and psychiatric disorders (Campbell et al., 2014). They also highlight the potential for therapeutic interventions by modulation of the kynurenine pathway.

Assas and colleagues discuss important aspects of neuro-immune communication and show how sensory fibers containing the neuropeptide calcitonin gene-related peptide (CGRP) shape the responses of macrophages, mast cells and other immune cells throughout the body and how these interactions contribute to immune defense and diverse inflammatory conditions (Assas et al., 2014). This neuropeptide and the c class nerve fibers that contain it thus form a key pathway for bi-directional neuroimmune interactions and could form a target for future neuroimmune based therapies.

Neuro-immune abnormalities not only affect adults and the elderly, but also play a role in diverse diseases that manifest in children. D'Angiulli et al. show that children in the Mexico City Metropolitan Area, who are chronically exposed to high concentrations of air pollutants, present with increased amounts of inflammatory mediators along with accumulation of misfolded

proteins in the cerebrospinal fluid (Calderon-Garciduenas et al., 2013). They propose that environmental factors could mediate detrimental actions in the developing brain.

Paul Ashwood and colleagues report that the behavioral characteristics, including social deficits, repetitive grooming behavior and atypical vocalizations, observed in BTBR T+tf/J mice are associated with the development of an inflammatory macrophage phenotype in this strain (Onore et al., 2013). They suggest that such a relationship between elevated inflammatory burden and repetitive grooming behavior may have relevance to the repetitive and stereotyped behavior characteristic of autism since many Autistic children also present with an increased inflammatory profile. Goyal and Miyan review the possible role of neuro-immune abnormalities in autism (Goyal and Miyan, 2014). They highlight the influence of environmental factors on the abnormal neurological, immunological, and neuroimmunological functions reported in Autistic children and discuss how these interactions can lead to or exacerbate autism spectrum disorder. Their discussion links poor development of the neuroimmune system to vulnerability to these environmental challenges and the consequential effects on the brain and its functions.

We hope that the articles presented in this research topic give thought-provoking and valuable insight into some of the important aspects of brain-immune interactions. Neuro-immune processes are likely to contribute to diverse pathologies in both the periphery and the brain leading to complex human diseases that affect millions of people worldwide. Understanding mechanisms of neuro-immune interactions could help to find appropriate therapies to some of these conditions.

REFERENCES

Ader, R., Felten, D., and Cohen, N. (1990). Interactions between the brain and the immune system. *Annu. Rev. Pharmacol. Toxicol.* 30, 561–602. doi: 10.1146/annurev.pa.30.040190.003021

Assas, B. M., Pennock, J. I., and Miyan, J. A. (2014). Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. *Front. Neurosci.* 8:23. doi: 10.3389/fnins.2014.00023

Calderon-Garciduenas, L., Cross, J. V., Franco-Lira, M., Aragon-Flores, M., Kavanaugh, M., Torres-Jardon, R., et al. (2013). Brain immune interactions and air pollution: macrophage inhibitory factor (MIF), prion cellular protein (PrP(C)), Interleukin-6 (IL-6), interleukin 1 receptor antagonist (IL-1Ra), and interleukin-2 (IL-2) in cerebrospinal fluid and MIF in serum differentiate urban children exposed to severe vs. low air pollution. *Front. Neurosci.* 7:183. doi: 10.3389/fnins.2013.00183

Campbell, B. M., Charych, E., Lee, A. W., and Moller, T. (2014). Kynurenines in CNS disease: regulation by inflammatory cytokines. *Front. Neurosci.* 8:12. doi: 10.3389/fnins.2014.00012

Denes, A., Pradillo, J. M., Drake, C., Buggey, H., Rothwell, N. J., and Allan, S. M. (2013). Surgical manipulation compromises leukocyte mobilization responses and inflammation after experimental cerebral ischemia in mice. *Front. Neurosci.* 7:271. doi: 10.3389/fnins.2013.00271

Denes, A., Thornton, P., Rothwell, N. J., and Allan, S. M. (2010). Inflammation and brain injury: acute cerebral ischaemia, peripheral and central inflammation. *Brain Behav. Immun.* 24, 708–723. doi: 10.1016/j.bbi.2009.09.010

Deretzi, G., Kountouras, J., Polyzos, S. A., Zavos, C., Giartza-Taxidou, E., Gavalas, E., et al. (2011). Gastrointestinal immune system and brain dialogue implicated in neuroinflammatory and neurodegenerative diseases. *Curr. Mol. Med.* 11, 696–707. doi: 10.2174/156652411797536660

Elenkov, I. J., Wilder, R. L., Chrousos, G. P., and Vizi, E. S. (2000). The sympathetic nerve–an integrative interface between two supersystems: the brain and the immune system. *Pharmacol. Rev.* 52, 595–638.

Garcia-Bonilla, L., Benakis, C., Moore, J., Iadecola, C., and Anrather, J. (2014). Immune mechanisms in cerebral ischemic tolerance. *Front. Neurosci.* 8:44. doi: 10.3389/fnins.2014.00044

Geenen, V., Bodart, G., Henry, S., Michaux, H., Dardenne, O., Charlet-Renard, C., et al. (2013). Programming of neuroendocrine self in the thymus and its defect in the development of neuroendocrine autoimmunity. *Front. Neurosci.* 7:187. doi: 10.3389/fnins.2013.00187

Goyal, D. K., and Miyan, J. A. (2014). Neuro-immune abnormalities in autism and their relationship with the environment: a variable insult model for autism. *Front. Endocrinol. (Lausanne)* 5:29. doi: 10.3389/fendo.2014.00029

Heneka, M. T., Kummer, M. P., and Latz, E. (2014). Innate immune activation in neurodegenerative disease. *Nat. Rev. Immunol.* 14, 463–477. doi: 10.1038/nri3705

Iadecola, C., and Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nat. Med.* 17, 796–808. doi: 10.1038/nm.2399

Kamimura, D., Yamada, M., Harada, M., Sabharwal, L., Meng, J., Bando, H., et al. (2013). The gateway theory: bridging neural and immune interactions in the CNS. *Front. Neurosci.* 7:204. doi: 10.3389/fnins.2013.00204

Moreno-Smith, M., Lutgendorf, S. K., and Sood, A. K. (2010). Impact of stress on cancer metastasis. *Future Oncol.* 6, 1863–1881. doi: 10.2217/fon.10.142

Onore, C. E., Careaga, M., Babineau, B. A., Schwartzer, J. J., Berman, R. F., and Ashwood, P. (2013). Inflammatory macrophage phenotype in BTBR T+tf/J mice. *Front. Neurosci.* 7:158. doi: 10.3389/fnins.2013.00158

Rivest, S. (2009). Regulation of innate immune responses in the brain. *Nat. Rev. Immunol.* 9, 429–439. doi: 10.1038/nri2565

Theoharides, T. C., Asadi, S., and Patel, A. B. (2013). Focal brain inflammation and autism. *J. Neuroinflammation* 10:46. doi: 10.1186/1742-2094-10-46

Wraith, D. C., and Nicholson, L. B. (2012). The adaptive immune system in diseases of the central nervous system. *J. Clin. Invest.* 122, 1172–1179. doi: 10.1172/JCI58648

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 October 2014; accepted: 09 November 2014; published online: 03 December 2014.

Citation: Denes A and Miyan JA (2014) Brain-immune interactions in health and disease. Front. Neurosci. ***8****:382. doi: 10.3389/fnins.2014.00382*

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 07 March 2014
doi: 10.3389/fendo.2014.00029

Neuro-immune abnormalities in autism and their relationship with the environment: a variable insult model for autism

Daniel K. Goyal* and Jaleel A. Miyan

Faculty of Life Sciences, The University of Manchester, Manchester, UK

Edited by:
Adam Denes, The University of Manchester, UK

Reviewed by:
Cynthia L. Bethea, Oregon Health and Science University, USA
Jozsef Halasz, Vadaskert Child Psychiatry Hospital, Hungary

****Correspondence:***
Daniel K. Goyal, Faculty of Life Sciences, The University of Manchester, AV Hill Building, Oxford Road, Manchester M13 9PT, UK
e-mail: danielgoyal@doctors.org.uk

Autism spectrum disorder (ASD) is a heterogeneous condition affecting an individual's ability to communicate and socialize and often presents with repetitive movements or behaviors. It tends to be severe with less than 10% achieving independent living with a marked variation in the progression of the condition. To date, the literature supports a multifactorial model with the largest, most detailed twin study demonstrating strong environmental contribution to the development of the condition. Here, we present a brief review of the neurological, immunological, and autonomic abnormalities in ASD focusing on the causative roles of environmental agents and abnormal gut microbiota. We present a working hypothesis attempting to bring together the influence of environment on the abnormal neurological, immunological, and neuroimmunological functions and we explain in brief how such pathophysiology can lead to, and/or exacerbate ASD symptomatology. At present, there is a lack of consistent findings relating to the neurobiology of autism. Whilst we postulate such variable findings may reflect the marked heterogeneity in clinical presentation and as such the variable findings may be of pathophysiological relevance, more research into the neurobiology of autism is necessary before establishing a working hypothesis. Both the literature review and hypothesis presented here explore possible neurobiological explanations with an emphasis of environmental etiologies and are presented with this bias.

Keywords: autism spectrum disorder, neuro-immune, environment, gut microbiota, neuroinflammation

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder of unknown etiology. Recent evidence suggests a strong environmental component (1) and persistent neuroinflammation (2–5). Within the phenology of ASD and associated disorders, the subjectivity involved in attributing an infant or toddler with introversion (or being in one's own world, autism) is fraught with difficulty. The difficulty is not whether such behavioral abnormalities represent a neurobiological illness – consensus is for an organic brain disorder – the challenge stems from the wide-ranging possibilities underlying the visible disease (6, 7). A secondary obstacle to the adequate identification of disease process in ASD patients pertains to scientific disparity. Research efforts have focused more on the genetic aspects of ASD than on environmental factors over the previous 15 years (8). Consequently, several important factors have impinged upon progress for ASD sufferers and those at risk.

The fixation on genetics led to reprioritization by medical staff and displacement of non-genetic scientific researchers. Many clinicians awaited the genetic answer and the promise of targeted, scientifically originated treatment. Conveying the certainty of the scientific consensus at the time, clinicians are now faced with the same patients and a different, almost polar growing certainty: there are likely to be prognostic factors one can mitigate (9, 10).

Previous twin studies suggested a predominant genetic component; however, these studies were poorly designed and had weak power (16, 17). A recent twin study published in July 2011 was well-designed with a substantial statistical power. One hundred ninety-four twin groups were studied and clinically evaluated prior to statistical analysis. Probandwise concordance for monozygotic twins was 77% (95% CI, 65–86%) for 45 male pairs and 50% (95% CI, 16–84%) for 9 female pairs. Concordance rates for dizygotic twins were 31% (95% CI, 16–46%) and 36% (95% CI, 11–60%) for 45 male and 13 female pairs, respectively. The study concluded: autism has substantial environmental factors, and indeed the environmental factors were of more significance than genetic factors (1).

There has been another compounding factor. Diagnostic labeling has changed substantially. Since its discovery in the late 1930s, autism has gradually become the diagnosis of choice. It has replaced and superseded childhood schizophrenia and feeblemindedness and has encompassed within the spectrum, a host of neurodevelopmental disorders [for review see Ref. (11)]. This allowed a rational argument for the increase in prevalence, and as such a tempering of the strict scientific critique required.

Recent advancements in ASD research has led to a surge in research activity, in particular neuro-immune and environmental factors. Here, we present a view of ASD related to neurological, immunological, and neuroimmunological findings from the bias of an environmental etiology standpoint. We briefly discuss the pertinent literature concerning the frequently reported abnormal

gut microflora composition in ASD patients. Finally, based on the growing consensus in biological scientific evidence and clinical experience, we present the variable insult model of ASD with the aim of contributing further to a useful research direction for those suffering from ASD and for those faced with managing the condition.

EPIDEMIOLOGY

Autism spectrum disorder was first identified by Kanner in 1938 (12). Over the subsequent 10 years, Kanner discovered 50 further cases (13). Kanner subsequently reviewed the first 11 patients at 30-year follow-up. Only one known patient achieved employment (14). More recent evidence also suggests a high level of disability in affected individuals, with 60–75% achieving poor or very poor outcomes in adulthood (15).

Autism spectrum disorder case detection rates are now substantially higher – from 1 in 3000 reported in 1966 (including both autism and psychosis) (16), 1 in 150 in 8-year-olds in 2007 [Centre for Disease Control (CDC), (17)], and in 2012 a rate of 1 in 88 [CDC, (18)]. In UK, Cohen et al. described a prevalence rate of 1 in 64 (19).

MORBIDITY AND MORTALITY

Shavelle et al. investigated the mortality rate of ASD in over 13,000 patients between 1983 and 1997 (20) and found it to be more than twice that of neurotypical peers. Standardized mortality ratio (SMR) was estimated as 2.4. Certain causes carried significantly higher SMR (see **Table 1**). Similar mortality rates have been reported in other studies (21, 22) with a consistent increased mortality rate for ASD, and a substantially greater risk in female ASD patients. Whilst mental retardation predicted risk of early demise, those without mental retardation also had increased risk.

DISEASE PROGRESSION

There have been several studies evaluating diagnostic stability over time. Turner et al. reassessed 2-year-olds diagnosed with ASD at 4.5 years of age (23) and found no change in their diagnosis of ASD but did find that 20% of children worsened between 2 years of age and 4.5 years of age and 20% improved. Within the parameters addressed, 60% remained relatively stable. No reason was identified for the variation.

Levy et al. have recently reviewed the literature regarding long-term outcome in ASD finding cognitive improvement in 20–55%, cognitive stability in 20–70%, and cognitive loss in 10–15% (24). No reasons were identified for why some ASD patients suffer a progressive illness and others make some recovery. ASDs as a group carry a poorer prognosis than other developmental disorders in almost all domains (24).

SUMMARY OF EPIDEMIOLOGICAL FINDINGS

Even though ASD is associated with high health, social, and financial impacts, investigative epidemiology has been limited. Perhaps the premature acceptance of ASD as a genetic condition limited the power of epidemiological science beyond that of detection of cases [for review see Ref. (25)]. The Interagency Autism Coordinating Committee (IACC) and Centers for Autism and Developmental Disabilities Research and Epidemiology (CADDRE) Network are co-ordinating a large epidemiological study in the US: the study to explore early development (SEED) (26). This is in keeping with the responsibilities set out in the US through "The Combating Autism Act 2006."

Table 1 | Causes of death in ASD with moderate to severe retardation or none to mild retardation (in brackets).

Cause of death	Early childhood SMR (5–10 years)	Late childhood SMR (10–20 years)	Adulthood SMR (>20 years)
Drowning	90.6 (14.1)	n/s	n/s
Digestive	n/s	40.8	5.9
Respiratory	n/s	24.5	9.4
Cancer	n/s	12.0 (3.8)	2.4 (1.6)
Nervous and sense	n/s	6.4 (15.9)	4.1
Seizures	n/s	n/s	30.8 (33.1)
Cardiovascular	n/s	n/s	3.7 (2.2)

Adapted from Ref. (20). SMR, standardized mortality ratio; n/s, no significant increase in SMR found in either group.

IMMUNE ABNORMALITIES AND NEUROINFLAMMATION IN ASD

Perhaps one of the most substantive studies in the last decade was conducted at the John Hopkins Institute, and involved an analysis of autopsy specimens and cerebrospinal fluid (CSF) samples from affected individuals and controls (2). The results indicated a neuroinflammatory response, regardless of age (in patients between 5 and 46 years of age), involving excess microglial activation and increased pro-inflammatory cytokine profiles. The study carries high statistical significance [for review of study, see Ref. (27)] and indicates an inflammatory state probably exists in the brains of these patients. Similar findings were found in a more recent autopsy study of microglia densities in fronto-insular and visual cortices of patients with ASD versus controls, and found a statistically significant ($p \leq 0.0002$) increase in microglial density in both regions (4). Other immune abnormalities have also been found indicating an inflammatory state. Transforming growth factor beta 1 (TGF-β1) is reduced in ASD cohorts versus controls and individuals with other developmental disorders and was found to be inversely proportional to behavior outcomes (irritability, lethargy, stereotypy, and hyperactivity) as well as with levels of social adaptability (28).

Natural killer cells (NK cells) are abnormal in sub-groups of ASD. NK cells respond to macrophage-derived cytokines and are essential in tumor prevention and host anti-viral activity. Enstrom et al. (29) found a significant reduction in NK cell cytotoxicity and a 2.5-fold increase in KSP-37, an NK gene normally induced during active viral infection. They concluded that ASD patients have activated but resting NK cells with increased levels of cytolytic proteins and an altered response to stimulation with changes in gene expression (29). Supporting these findings, cancer mortality rates are higher in ASD (20), and the only identified risk factor for mortality associated with the recent H1N1 outbreak was developmental delay (30). Both of these findings suggest immune dysfunction in

ASD, and either or both of these findings could be linked with the NK cell abnormalities identified by Enstrom et al. (29).

There have been studies making correlations between measures of immune functions and cytokine profiles with behavioral measures in ASD (**Table 2**). Significant correlations were shown between certain behavioral indices and the chemokine's, macrophage chemoattractant MCP-1, macrophage inflammatory protein (MIP)-1β, eotaxin, and "regulated upon activation normal T-cell expressed and secreted" factor (RANTES) (31). RANTES was associated with lethargy, stereotypy, and hyperactivity. Eotaxin was associated with hyperactivity, visual perception, fine motor control, expressive language, communication and daily living skills, and socialization. MCP-1 was associated with visual perception. These associations, if proven to be functional, raise many questions pertaining to the immune system's connectivity to the nervous system and involvement in neurobehavioral illnesses (for summary of immunological findings relating to behavior in ASD, see **Table 2**). Of importance here is the probability of immune involvement in the core features of ASD. These findings also raise the possibility of assessing behavioral changes in ASD through a quantitative measure.

NEUROLOGICAL ABNORMALITIES IN ASD

With the exception of neuroinflammatory changes, most reported neurobiological abnormalities in ASD are inconsistent.

Structurally, abnormalities have been described in the cerebellum, hippocampus, amygdala, and insular cortex (32). Abnormal brain volume has also been identified [for review see Ref. (33)]. A

Table 2 | Behavior and immune functions in ASD [adapted from Ref. (84)].

Studies	*n*	Age	Assessment method	Immune measure	Behavior measure
Ashwood et al. (28)	143	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, and ABC	Plasma levels of active TGFβ1	Lower TGFβ1 levels were associated with lower adaptive behaviors and worse behavioral symptoms
Iwata et al. (89)	37	20–25	ADI-R	Plasma levels of P-selectin	Lower levels of P-selectin associated with poor social development
Heuer et al. (90)	271	2–5	ADI-R, ADOS, and ABC	IgG levels in plasma	Decreased IgG associated with increased aberrant behaviors
Grigorenko et al. (91)	1059	n/s	ADI-R and ADOS	Genotyping of the MIF gene and plasma levels of MIF ($n = 20$)	Plasma MIF levels were positively correlated with worse scores on ADOS for social impairment and imaginative skills
Onore et al. (92)	60	2–5	ADOS, ADI-R, MSEL, VABS, and ABC	Induced cytokine response to PHA	Negative correlation between PHA induced IL-23 production and sociability scores of the ADOS
Enstrom et al. (93)	30	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, and ABC	Monocyte TLR ligand stimulation	More impaired social behaviors and non-verbal communication are associated with increased production of IL-1β and IL-6 after TLR4 stimulation
Ashwood et al. (94)	139	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, and ABC	Induced cytokine response to PHA and LPS	Pro-inflammatory or TH1 cytokines were associated with greater impairments in core features of ASD as well as aberrant behaviors; GM-CSF and TH2 cytokines were associated with better cognitive and adaptive function
Goines et al. (95)	466	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, and ABC	Antibodies directed against a 45 or 62 kDa cerebellum protein	Children with antibodies directed against a 45-kDa cerebellum protein had increased lethargy and stereotypy; children with antibodies against a 62-kDa cerebellum protein showed increased aberrant behaviors on the VABS composite standard score
Kajizuka et al. (96)	62	6–19	ADI-R	Serum levels of PDGF	Increased serum levels of PDGF-BB homodimers positively associated with increased restricted, repetitive, and stereotyped patterns of behavior and interests
Ashwood et al. (31)	175	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, and ABC	Plasma chemokines CCL2, CCL5, and eotaxin	Plasma chemokine levels associated with higher aberrant behavior scores and more impaired developmental and adaptive function
Ashwood et al. (97)	223	2–5	ADI-R, ADOS, SCQ, VABS, MSEL, and ABC	Plasma levels of cytokines IL-1β, IL-6, IL-8, and IL-12p40	Elevated cytokine levels in plasma were associated with more impaired communication and aberrant behaviors
Ross et al. (98)	16	3-31	ADI-R	GM-CSF, INFγ, IL-12p70, IL-1β, IL-6, IL-8, TNFα, and IL-10	Elevation of cytokines correlated with autistic symptoms in patients with 22q11.2 deletion syndrome

meta-analysis reported on an average of 13% smaller brain volume at birth, an average of 10% larger brain volume at 1 year of age than controls, and 2% larger in adolescence (33). An increase in gray matter with a reduced unit density has been quite reliably identified in this cohort (33). CSF volume has also been reported to be increased with enlarged ventricles (34) and mini-columnar size is decreased (35). It has been proposed that such structural variation may affect adaptation and hence learning, and may account for the heterogeneity and wide-ranging functional deficits seen in ASD (36). Disordered neural connectivity has been discussed for some time (37). The evidence supports under-connectivity between sensory cortices and association cortices in moderate to severe ASD, essentially leading to a failure to assimilate sensory information into a working environmental context, and a lack of connectivity of associative cortices to the frontal cortex in higher functioning autism (38). This helps explain associated learning difficulties in low functioning autism, and the poor fine motor control and impaired imitation identified in higher functioning autism (39–41).

AUTONOMIC DYSFUNCTION

Autonomic involvement in ASD has been widely reported for over 30 years (42–52). A recent controlled trial explored in detail the nature and type of autonomic involvement (49). Real-time variability together with continuous monitoring of blood pressure and breathing rhythms were assessed in an ASD cohort versus controls. Over 80% of the ASD cohorts were found to have a reduced vagal tone, highly suggestive of low central parasympathetic activity and, significantly, in a separate study, vagal tone in the neonate was found to predict neurodevelopmental outcome more accurately than birth weight, socio-economic status, or co-morbid medical conditions (50). Given that the autonomic nervous system (ANS) is responsible for the majority of sensory information received by the central nervous system, any disruption to the ANS is likely to have wide-ranging effects on higher cortical development. In a longitudinal follow-up study, Goytag et al. examined the order of cortical development using repeat MRI and concluded: higher-order association cortices mature only after lower-order somatosensory and visual cortices, the functions of which they integrate, are developed (53). The development of a normal parasympathetic tone is thus likely to be crucial for adequate neurodevelopment [for review see Ref. (50)]. More research is required.

ENVIRONMENTAL ASPECTS OF ASD

Hertz-Picciotto et al. provide a detailed review of developmental immunotoxicity (DIT) in relation to neurodevelopment disorders (54). Xenobiotic exposure in early life may lead to altered immune function throughout life, a persistent neuroinflammatory response and systemic immune dysregulation, and the possibility of a neurobehavioral manifestation of the disease (54).

The type of immune dysfunction relates to the type of xenobiotic involved and the timing of xenobiotic exposure with dose-dependent effects not as applicable in the developing immune system but specific neurological development depends on signaling from the immune system (55). Such conveyance of environmental state to the nervous system from the immune system is advantageous and essential, but is incompatible with adequate neurodevelopment in poor environmental conditions. Whether there is a single type of xenobiotic involved in the etiology of ASD or whether the pathophysiology involves exposure to general environmental pollution remains a keenly researched area. Pesticides, flame retardants, heavy metals, traffic fumes, and endotoxins have all been implicated (56–61). The apparent lack of consistency is further compounded by difficulties in measuring chronic toxicity and toxin-induced disruption particularly if the xenobiotic exposure is no longer present.

Dietert et al. describes several similarities between early-life immune insults (ELII), including DIT and ASD with gender differences, time-windows for immune development, and the corresponding variable presentation in both ELII and ASD making a compelling argument (62). Studying DIT and indeed developmental neurotoxicity requires functional measures and a history of significant exposure. Complicating matters further is the modest, but significant genomic variation in xenobiotic metabolism and hence resistance or vulnerability to environmental exposures.

ABNORMALITIES IN GUT MICROFLORA IN ASD

Abnormal clostridia species have been found repeatedly in ASD (63–67). The theory of clostridia involvement was postulated by Bolte in 1998 who suggested that clostridia toxin adversely affected neurotransmitter function that could result in neurobehavioral changes presenting as autism (68). Supporting this hypothesis, Parracho et al. outlined robust measures of microflora abnormalities in ASD cases suffering from bowel problems using PCR analysis and found a clear and consistent abnormality in the clostridia species present in ASD sufferers versus controls. *Clostridium histolytica* were found in higher levels in the ASD group versus healthy unrelated controls ($p < 0.01$) and healthy related controls ($p < 0.05$) (65).

A clinical trial was carried out to assess the bowel and behavioral impact of anti-microbial therapy directed against these abnormal clostridia species (69). Oral vancomycin was used for 6 weeks. Behavioral measurements were carried out before and after, as well as clinical assessment of bowel symptoms. The numbers were low but the response to intervention was reported as statistically significant. 8 of the 10 patients studied improved in terms of behavior and bowel symptoms with some scoring within the neurotypical range. Discontinuation of vancomycin after the 6-week trial period led to a gradual regression in bowel and behavioral symptoms in all participants (69) suggesting that gut environment gives preference to these abnormal species. As yet, there has been no investigation of the combined approach of anti-microbial therapy and other interventions targeted at altering microbiota composition.

Williams et al. recently reported consistently abnormal Firmicutes to Bacteriodetes ratios from biopsy specimens in children with ASD versus inflammatory bowel disease (IBD) controls. This was linked to reduced disaccharidases (starch digesting enzymes), which in the same study were also found to be low in the ASD group. Williams et al. postulated a link between high carbohydrate transit to the large intestine in ASD leading to alteration in the proportion of Firmicutes to Bacteriodetes. The appearance of this "compositional dysbiosis" was highly correlated in the ASD group with Firmicutes to Bacteriodetes ratio of 31:69 (versus controls

27:73) in the ileal biopsies ($p < 0.0006$) and 32:68 (versus controls 25:75) in the cecal biopsies ($p < 0.022$) (66).

Although microflora are known to alter host immune function, including cytokine production [for review see Ref. (70–73)], to our knowledge there has, to date, been no investigation of the relationship between abnormal microflora and cytokine production in ASD, although a few studies have examined cytokines in ASD patients with bowel symptoms and found positive correlations (72–74).

DISCUSSION

It is the wide heterogeneity of ASD that poses the greatest challenge. Identifying a common pathophysiology is hampered by such diversity as is the identification of management strategies, both behavioral and medical. Equally those faced with patient care often struggle to discern the range of presentations and the impact this has on management. ASD shares simply, a marked impairment with any of the faculties required for social integration; this can present with a lethargic, disinterested child or an agitated, distracted child or any number of features leading ultimately to impaired social integration. It may be that in order to determine treatment response, we must first delineate/categorize treatable groups. Indeed if some form of environmental insult occurs early on in development (be it infection, toxicant, or other environmental stressor), then it may not only cause variable manifestations based on timing, nature, and genomic individuality, it may also leave no discernible or at least easily discernible trace. It may, as Hertz-Piciotta et al. (54) and Dietert et al. (62) suggest, merely be an event that primes or disrupts a critical window in development. Given the wide-ranging heterogeneity of the disorder and the many faculties required for social integration, it may be neither the insulting agent, the timing, the genomic vulnerability nor the system(s) affected that remain static or, when ASD is taken as a single consequential expression, statistically identifiable.

Within the developing neurological, immunological, and neuroimmunological systems, there is vulnerability to environmental insult. Depending on the nature, timing, and duration of insult, neurological, immunological, or neuroimmunological abnormalities may predominate, and the relative proportion each system is affected will vary accordingly (see **Figure 1**).

Socialization and speech are complex neurological functions. ASD may represent impairment in any system/faculty required to facilitate such complex neurological functions. There is likely ample opportunity for an environmental insult to disrupt one or more of the mechanisms leading to the impairment of the higher-order processes of social integration and along the way disrupt a number of other physiological mechanisms that may contribute or indeed cause the additional and variable behavioral manifestations within the spectrum of disease. As troublesome as the notion may be, and granted there will be reasonable pathophysiological correlations identified particularly within sub-groups, the greatest commonality in ASD may be etiological, and even then it may merely be a trend in human–environment relationship versus an exact noxious substance.

Similar hypotheses have been presented by others: e.g., Dietert et al. (47), Hertz-Picotta et al. (46), Goines and Ashwood (75), and Unwin et al. (76). The central theme is the presence of a variable insult leading to the variable presentation of ASD. In recognition of these previous studies, the hypothesis presented here is referred to as the variable insult model of ASD. Such hypotheses are critical to providing a framework to identify sub-types within the ASD group, building in some predictability both for researchers and for those faced with clinical management. Such sub-typing can be based on the dominant system pathophysiology involved or one can attempt clinical classification. **Table 3** is an example of such an attempt at clinical sub-typing based on the variable insult model of ASD.

The sub-categories presented in **Table 3** are broad and overlap considerably. More specific sub-typing seems probable, perhaps relating to the intensity of the insult and perhaps more specifically to the offending agent, as suggested recently by Unwin et al. (76). Already abnormal RNA transcription has been identified in ASD children correlating with environmental toxicants versus controls with similar levels of toxicants (77, 78). The transcription abnormalities are specific to the toxicant, raising the possibility of different etiological agents triggering different initial pathophysiological mechanisms sharing only the secondary consequences. The factors involved in the different gene expression in ASD, whether they be linked to genomic individuality, previous exposure, some kind of immunological priming, or abnormal GI flora, raises interesting questions, but in these current considerations the mere difference in RNA transcription between different xenobiotics and also between ASD patients versus control groups raises important questions about accurate delineation of sub-types and the different pathophysiological mechanisms involved in the eventual ASD outcome.

Variable pathophysiological pathways leading to ASD seem likely, and recent advancements in scientific techniques carry the capacity to differentiate each pathway with relevance to the prevention and clinical management of the condition. For example, PCR analysis of GI microflora continues to reveal deeper insights into the common GI abnormalities prevalent in ASD (65, 66, 81). Evidence demonstrates the importance of such microflora on immune and neurological function, and the evolution of GI microflora composition over the first few years of life (82, 83). The compositional dysbiosis discovered by Williams et al. (66) in ASD patients may reflect another common manifestation of ASD due perhaps to similar complexities as is involved in social integration with genomic, neurological, immunological, and neuroimmunological systems required to select and regulate the GI microflora. This may explain the frequently reported presence of abnormal species in ASD and the diversity of such abnormal microflora/pathogens [for review see Ref. (65, 81)]; the selection and regulation processes are also part of the developmental process and are vulnerable to a variety of insults at a variety of levels. ASD diagnosis may be more scientifically sound should it move toward a formulation including environmental exposure, genomic vulnerability, and the identification of the system(s) pathophysiology with treatment interventions based on such measurable criteria. Animal models can serve such ever increasing sub-categorization, modeled to reflect each category and utilized to identify novel therapeutic interventions at specific groups. Without such an appreciation of the variability associated with ASD, it may be difficult to achieve

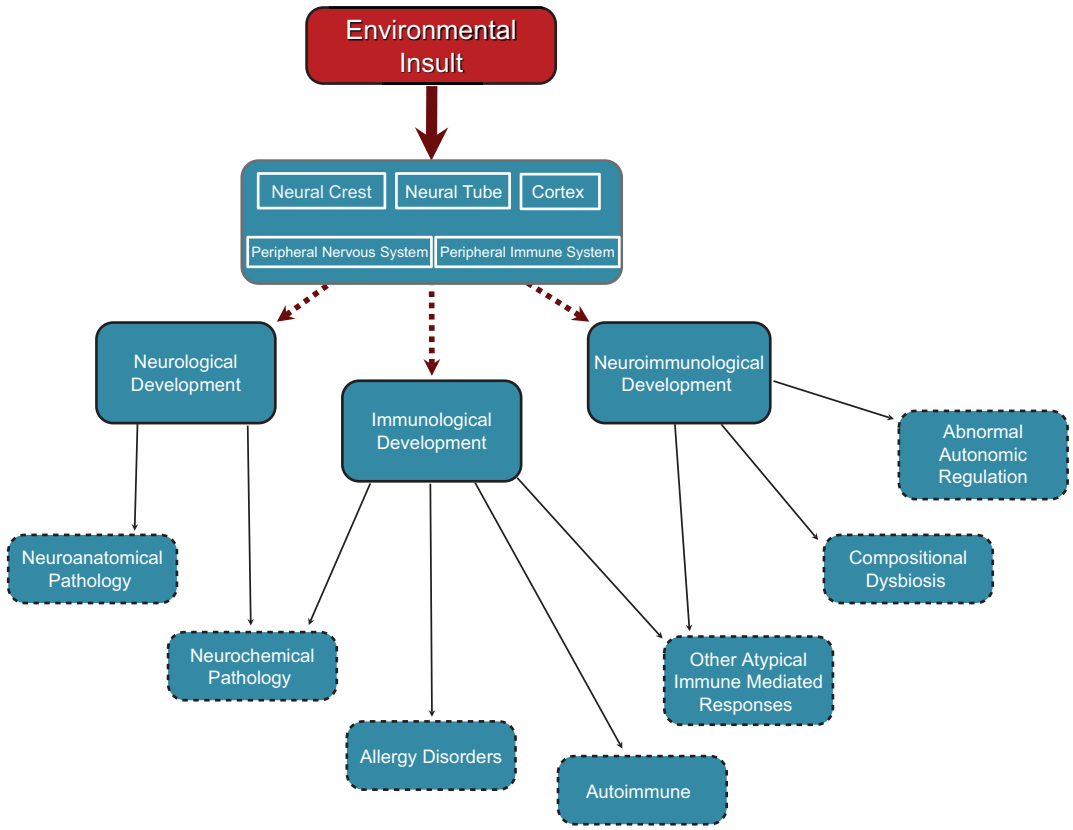


FIGURE 1 | Cascade of effects originating in neurodevelopmental insults occurring at different points in development. Insults can occur at any point in neurodevelopment from embryonic through to juvenile and perhaps even adulthood. Insults to neural crest and neural tube development in the embryo would have structural and functional outcomes in all peripheral neural elements including autonomic (sympathetic, parasympathetic), peripheral sensory system, and enteric (gut) nervous system. Neural tube effects might also produce changes in central autonomic functions and/or set points for physiological control systems resident in the hypothalamus and brain stem autonomic nuclei. Factors affecting cortical development would have an impact on higher cortical functions including psychosocial, sensory processing, and motor functioning. Any of these neurodevelopmental effects would have cascading effects through the sensory and neuro-immune pathways to neurological, neuro-immune, and immune functions.

statistical significance in treatment trials targeted at specific deficits.

The variable insult hypothesis predicts diverse immune-related abnormalities, probability of poor GI microflora regulation, variable autonomic function with impaired/disordered autonomic reactivity, and variable neuroanatomical findings. Further, the variable insult model predicts that ASD animal models could be established through a variety of mechanisms. Valproate with an acute single dose administered gestationally at E12.5 leads to ASD symptomatology in rats (79), as does valproate administered in a sub-chronic dosing given between post-natal days P6–12, albeit the sub-chronic dosing may have more sensory issues (80). Unwin et al. (76) proposed that homogeneity may be found by identifying the etiological agent, and presents the differences in associative symptomatology in children with ASD who had either perinatal exposure to selective serotonin re-uptake inhibitor (SSRI) or who had low birth weight (LBW). The SSRI group seemed to have more gastrointestinal disturbance, although it was not clear whether this was a depression effect or drug effect, and the LBW group had more sleep and breathing disturbance. Within the variable insult model of ASD, SSRI exposure may represent greater disruption to the peripheral ANS, perhaps during neural crest formation and development, whereas the LBW group may have suffered more pronounced central autonomic disruption. Difficulties with sensory processing may then affect both groups through different mechanisms with similar social outcomes.

If we start to look at the etiological agents contributing to the development of ASD, then perhaps we can start to find sub-groups. Such an approach brings more complexity to the clinical assessment of patients suffering from ASD but perhaps such a complex condition requires greater effort and more complex formulations prior to predicting prognosis and response to medical or behavioral management. If we go further still and delineate the various pathophysiologies that can lead to ASD symptomatology through animal and human experiments, then early identification of the system(s) requiring attention may be possible and may then better guide harm reduction strategies. Prevention remains the priority, but harm reduction through focused scientific investigation could reduce the burden of disease going forward. For example,

Table 3 | Broad clinical sub-categories of ASD.

	Prenatal – birth		Infant – early child (birth to 3 years)	
	Early acute insult	**Early chronic insult**	**Late acute insult**	**Late chronic insult**
Congenital abnormalities	++++	+++	+	+
Severe dysmotility	++++	+++	++	++
Sudden regression	+	++	++++	+++
Insidious regression	++	+++	+	++++
Early immune-related issues	+++	++++	++	++
Motor delays	++++	+++	+	+
Family history of autoimmunity	++	++	++++	+++
Gestational exposure	++++	+++	+	+
Early infancy exposure	+	++	++++	++++

The number of crosses indicates the severity/frequency of each measure relative to age of exposure and its duration. Early acute insult refers to a sudden or relatively sudden, usually marked exposure to xenobiotic, infection, or other environmental stressor during prenatal or gestational periods. Early chronic insult refers to a sustained usually moderate level of exposure over a period of months during prenatal and/or gestational period. Late acute insult refers to a sudden or relatively sudden, usually marked exposure to xenobiotic, infection, or other environmental stressor in infancy to early childhood (birth to 3 years). Late chronic insult refers to a sustained moderate level of exposure over a period of months during infancy to early childhood.

in Unwin et al.'s SSRI group, early intervention relating to GI pathology could allow correction of the dysfunctional peripheral autonomic input and thus permit greater sensory integration, and thus improved developmental outcome. Equally addressing the breathing dysrhythmias in the LBW group may improve autonomic reactivity improving both sensory integration and neuro-immune responsiveness. Elsewhere, we present clinical data to support such a possibility through improved autonomic function following management of co-morbid health conditions and the subsequent improvement in ASD symptoms.

CLINICAL APPLICATION

The pathophysiology of ASD remains largely unknown. Evidence so far suggests prognosis is not pre-determined and there is a dynamic component of the disease. The greater frequency of extra-CNS disease(s) (85–88), increased respiratory and GI-related childhood mortality, and substantially greater risk of progressive seizure disorders (20–22) together with the frequently reported neuroinflammatory changes (2–5) in ASD suggests ongoing disease activity, and whilst targeted treatment trials are awaited these tangible aspects of disease must be the focus of clinical intervention. Actively seeking extra-CNS disease in ASD and pro-actively managing such conditions, with the appreciation that presentation of such disease and response to intervention may not be typical or, whilst the pathophysiology remains ill-defined, predictable, is a pillar of harm reduction. Delineating sub-groups further will hopefully help predict the areas requiring further attention in each patient group.

SUMMARY

Autism spectrum disorder is a severe neurological condition with variable presentation, disease evolution, and variable, albeit generally poor, functional outcomes. Patients with ASD have greater risk of physical and mental health complications, and also a greater mortality. Neuroinflammation, peripheral immune abnormalities, and environmental factors have consistently been identified, further supporting the need for research that prioritizes disease prevention and harm reduction. The results from large epidemiological studies are awaited to identify potential key areas for this research.

Heterogeneity has been a significant barrier to successful intervention in ASD. It may be that the commonality of impaired social integration represents dysfunction of a wide variety of systems and faculties during a crucial developmental period required for the complexities of social integration and as such the commonality is merely etiological. Similar levels of intricacy may explain the propensity to abnormal acquisition of GI flora during an equally important microbiota developmental period. Here, we reviewed the most promising research findings to explain the diverse neurological, immunological, and neuroimmunological abnormalities in ASD within consideration of variable environmental insults. In our opinion, this provides a useful framework to understand and further explore this devastating neurodevelopmental condition.

REFERENCES

1. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, et al. Genetic heritability and shared environmental factors among twin pairs with autism. *Arch Gen Psychiatry* (2011) **76**:v1. doi:10.1001/archgenpsychiatry.2011.76
2. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann Neurol* (2005) **57**(1):67–81. doi:10.1002/ana.20315
3. Suzuki K, Sugihara G, Ouchi Y, Nakamura K, Futatsubashi M, Takebayashi K, et al. Microglial activation in young adults with autism spectrum disorder. *JAMA Psychiatry* (2013) **70**(1):49–58. doi:10.1001/jamapsychiatry.2013.272
4. Tetreault NA, Hakeem AY, Jiang S, Williams BA, Allman E, Wold BJ, et al. Microglia in the cerebral cortex in autism. *J Autism Dev Disord* (2012) **42**(12):2569–84. doi:10.1007/s10803-012-1513-0
5. Morgan JT, Chana G, Pardo CA, Achim C, Semendeferi K, Buckwalter J, et al. Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism. *Biol Psychiatry* (2010) **68**(4):368–76. doi:10.1016/j.biopsych.2010.05.024
6. Coleman M. Clinical review: medical differential diagnosis and treatment of the autistic syndrome. *Eur Child Adolesc Psychiatry* (1993) **2**(3):161–8. doi:10.1007/BF02125571
7. Steffenburg S. Neuropsychiatric assessment of children with autism: a population based study. *Dev Med Child Neurol* (1991) **33**(6):495–511. doi:10.1111/j.1469-8749.1991.tb14915.x
8. Lawler CP. The environmental for autism research: signs of improvement? *Environ Health Perspect* (2008) **116**(10):A416. doi:10.1289/ehp.12107
9. Interagency Autism Coordinating Committee. *2011 IACC Strategic Plan for Autism Spectrum Disorder Research*. Washington, DC: US Department of Health and Human Services (2011).
10. Combating Autism Act, US Public Law 109-416 (2006).
11. Coleman M, Gillberg C. *The Autisms*. New York: Oxford University Press (2012).
12. Kanner L. Autistic disturbances of affective contact. *Nervous child* (1943) **2**(3):217–50.
13. Kanner L. Problems of nosology and psychodynamics of early infantile autism. *Am J Orthopsychiatry* (1949) **19**(3):416–26. doi:10.1111/j.1939-0025.1949.tb05441.x
14. Kanner L. Follow-up study of eleven autistic children originally reported in 1943. *J Autism Dev Disord* (1971) **1**(2):119–45. doi:10.1007/BF01537953

15. Seltzer MM, Shattuck P, Abbeduto L, Greenberg JS. Trajectory of development in adolescents and adults with autism. *Ment Retard Dev Disabil Res Rev* (2004) **10**:234–47. doi:10.1002/mrdd.20038
16. Lotter V. Epidemiology of autistic conditions in young children: 1. Prevalence. *Soc Psychiatry* (1966) **1**:124–37. doi:10.1007/BF00584048
17. Kuehn BM. CDC: autism spectrum disorders common. *JAMA* (2007) **297**(9):940–940. doi:10.1001/jama.297.9.940
18. Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators, Centers for Disease Control and Prevention. Prevalence of autism spectrum disorders – autism and developmental disabilities monitoring network, 14 sites, United States, 2008. *MMWR Surveill Summ* (2012) **61**(3):1–19.
19. Baron-Cohen S, Scott FJ, Allison C, Williams J, Bolton P, Matthews FE, et al. Prevalence of autism-spectrum conditions: UK school-based population study. *Br J Psychiatry* (2009) **194**(6):500–9. doi:10.1192/bjp.bp.108.059345
20. Shavelle RM, Strauss DJ, Pickett J. Causes of death in autism. *J Autism Dev Disord* (2001) **31**(6):569–76. doi:10.1023/A:1013247011483
21. Gillberg C, Billstedt E, Sundh V, Gillberg IC. Mortality in autism: a prospective longitudinal community-based study. *J Autism Dev Disord* (2010) **40**(3):352–7. doi:10.1007/s10803-009-0883-4
22. Mouridsen SE, Brønnum-Hansen H, Rich B, Isager T. Mortality and causes of death in autism spectrum disorders. *Autism* (2008) **12**(4):403–14. doi:10.1177/1362361308091653
23. Turner LM, Stone WL. Variability in outcome for children with an ASD diagnosis at age 2. *J Child Psychol Psychiatry* (2007) **48**(8):793–802. doi:10.1111/j.1469-7610.2007.01744.x
24. Levy A, Perry A. Outcomes in adolescents and adults with autism: a review of the literature. *Res Autism Spectr Disord* (2011) **5**(4):1271–82. doi:10.1016/j.rasd.2011.01.023
25. Newschaffer CJ, Croen LA, Daniels J, Giarelli E, Grether JK, Levy SE, et al. The epidemiology of autism spectrum disorders. *Annu Rev Public Health* (2007) **28**:235–58.
26. Schendel DE, Diguiseppi C, Croen LA, Fallin MD, Reed PL, Schieve LA, et al. The study to explore early development (SEED): a multisite epidemiologic study of autism by the Centres for Autism and Developmental Disabilities Research and Epidemiology (CADDRE) Network. *J Autism Dev Disord* (2012) **42**:1–20. doi:10.1007/s10803-012-1461-8
27. Pardo CA, Vargas DL, Zimmerman AW. Immunity, neuroglia and neuroinflammation in autism. *Int Rev Psychiatry* (2005) **17**(6):485–95. doi:10.1080/02646830500381930
28. Ashwood P, Enstrom A, Krakowiak P, Hertz-Picciotto I, Hansen RL, Croen LA, et al. Decreased transforming growth factor beta1 in autism: a potential link between immune dysregulation and impairment in clinical behavioral outcomes. *J Neuroimmunol* (2008) **204**(12):149–53. doi:10.1016/j.jneuroim.2008.07.006
29. Enstrom AM, Lit L, Onore CE, Gregg JP, Hansen RL, Pessah IN, et al. Altered gene expression and function of peripheral blood natural killer cells in children with autism. *Brain Behav Immun* (2009) **23**(1):124–33. doi:10.1016/j.bbi.2008.08.001
30. Dalziel SR, Thompson JM, Macias CG, Fernandes RM, Johnson DW, Waisman Y, et al. Predictors of severe H1N1 infection in children presenting within Pediatric Emergency Research Networks (PERN): retrospective case-control study. *BMJ* (2013) **347**:f4836. doi:10.1136/bmj.f4836
31. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah IN, Van deWater J. Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *J Neuroimmunol* (2011) **232**(1):196–9. doi:10.1016/j.jneuroim.2010.10.025
32. Pardo CA, Eberhart CG. The neurobiology of autism. *Brain Pathol* (2007) **17**:434–47. doi:10.1111/j.1750-3639.2007.00102.x
33. Herbert MR. Large brains in autism: the challenge of pervasive abnormality. *Neuroscientist* (2005) **11**:417–40. doi:10.1177/0091270005278866
34. Hallahan B, Daly EM, McAlonan G, Loth E, Toal F, O'Brien F, et al. Brain morphometry volume in autistic spectrum disorder: a magnetic resonance imaging study of adults. *Psychol Med* (2009) **39**(02):337–46. doi:10.1017/S0033291708003383
35. Casanova M, Buxhoeveden D, Switala A, Roy E. Minicolumnar pathology in autism. *Neurology* (2002) **58**(3):428–32. doi:10.1212/WNL.58.3.428
36. Casanova MF. The significance of minicolumnar size variability in autism: a perspective from comparative anatomy. In: Zimmerman AW, editor. *Autism*. Totowa, NJ: Humana Press (2008) p. 349–60. doi:10.1007/978-1-60327-489-0_16
37. Minshew NJ, Payton JB. New perspectives in autism, Part II: the differential diagnosis and neurobiology of autism. *Curr Probl Pediatr* (1988) **18**:613–94. doi:10.1016/0045-9380(88)90017-5
38. Minshew NJ, Williams DL. The new neurobiology of autism. *Arch Neurol* (2007) **64**:945–50. doi:10.1001/archneur.64.7.945
39. Baird AD, Scheffer IE, Wilson SJ. Mirror neuron system involvement in empathy: a critical look at the evidence. *Soc Neurosci* (2011) **6**(4):327–35. doi:10.1080/17470919.2010.547085
40. Gowen E, Hamilton A. Motor abilities in autism: a review using a computational context. *J Autism Dev Disord* (2013) **43**(2):323–44. doi:10.1007/s10803-012-1574-0
41. Gowen E. Imitation in autism: why action kinematics matter. *Front Integr Neurosci* (2012) **6**:117. doi:10.3389/fnint.2012.00117
42. Hutt C, Forrest SJ, Richer J. Cardiac arrhythmia and behaviour in autistic children. *Acta Psychiatr Scand* (1975) **51**(5):361–72. doi:10.1111/j.1600-0447.1975.tb00014.x
43. Kootz JP, Cohen DJ. Modulation of sensory intake in autistic children: cardiovascular and behavioral indices. *J Am Acad Child Psychiatry* (1981) **20**(4):692–701. doi:10.1097/00004583-198102000-00002
44. Kootz JP, Marinelli B, Cohen DJ. Modulation of response to environmental stimulation in autistic children. *J Autism Dev Disord* (1982) **12**(2):185–93. doi:10.1007/BF01531308
45. Zahn TP, Rumsey JM, Van Kammen DP. Autonomic nervous system activity in autistic, schizophrenic, and normal men: effects of stimulus significance. *J Abnorm Psychol* (1987) **96**(2):135. doi:10.1037/0021-843X.96.2.135
46. James AL, Barry RJ. Respiratory and vascular responses to simple visual stimuli in autistics, retardates and normals. *Psychophysiology* (1980) **17**(6):541–7. doi:10.1111/j.1469-8986.1980.tb02294.x
47. Cohen DJ, Johnson WT. Cardiovascular correlates of attention in normal and psychiatrically disturbed children: blood pressure, peripheral blood flow, and peripheral vascular resistance. *Arch Gen Psychiatry* (1977) **34**(5):561. doi:10.1001/archpsyc.1977.01770170071006
48. Palkovitz RJ, Wiesenfeld AR. Differential autonomic responses of autistic and normal children. *J Autism Dev Disord* (1980) **10**(3):347–60. doi:10.1007/BF02408294
49. Ming X, Julu PO, Brimacombe M, Connor S, Daniels ML. Reduced cardiac parasympathetic activity in children with autism. *Brain Dev* (2005) **27**(7):509–16. doi:10.1016/j.braindev.2005.01.003
50. Field T, Diego M. Vagal activity, early growth and emotional development. *Infant Behav Dev* (2008) **31**(3):361–73. doi:10.1016/j.infbeh.2007.12.008
51. Kushki A, Drumm E, Pla Mobarak M, Tanel N, Dupuis A, Chau T, et al. Investigating the autonomic nervous system response to anxiety in children with autism spectrum disorders. *PLoS One* (2013) **8**(4):e59730. doi:10.1371/journal.pone.0059730
52. James AL, Barry RJ. Cardiovascular and electrodermal responses to simple stimuli in autistic, retarded and normal children. *Int J Psychophysiol* (1984) **1**(2):179–93. doi:10.1016/0167-8760(84)90037-0
53. Gogtay N, Giedd JN, Lusk L, Hayashi KM, Greenstein D, Vaituzis AC, et al. Dynamic mapping of human cortical development during childhood through early adulthood. *Proc Natl Acad Sci U S A* (2004) **101**(21):8174. doi:10.1073/pnas.0402680101
54. Hertz-Picciotto I, Park HY, Dostal M, Kocan A, Trnovec T, Sram R. Prenatal exposures to persistent and non-persistent organic compounds and effects on immune system development. *Basic Clin Pharmacol Toxicol* (2008) **102**(2):146–54. doi:10.1111/j.1742-7843.2007.00190.x
55. West LJ. Defining critical windows in the development of the human immune system. *Hum Exp Toxicol* (2002) **21**:499–505. doi:10.1191/0960327102ht288oa
56. Volk HE, Hertz-Picciotto I, Delwiche L, Lurmann F, McConnell R. Residential proximity to freeways and autism in the CHARGE study. *Environ Health Perspect* (2011) **119**(6):873. doi:10.1289/ehp.1002835
57. Hertz-Picciotto I, Bergman A, Fängström B, Rose M, Krakowiak P, Pessah I, et al. Polybrominated diphenyl ethers in relation to autism and developmental delay: a case-control study. *Environ Health* (2011) **10**:1. doi:10.1186/1476-069X-10-1
58. Toms LM, Sjödin A, Harden F, Hobson P, Jones R, Edenfield E, et al. Serum polybrominated diphenyl ether (PBDE) levels are higher in children (25 years

of age) than in infants and adults. *Environ Health Perspect* (2009) **117**(9):1461. doi:10.1289/ehp.0900596
59. Roberts EM, English PB, Grether JK, Windham GC, Somberg L, Wolff C. Maternal residence near agricultural pesticide applications and autism spectrum disorders among children in the California Central Valley. *Environ Health Perspect* (2007) **115**(10):1482. doi:10.1289/ehp.10168
60. Eskenazi B, Rosas LG, Marks AR, Bradman A, Harley K, Holland N, et al. Pesticide toxicity and the developing brain. *Basic Clin Pharmacol Toxicol* (2008) **102**(2):228–36. doi:10.1111/j.1742-7843.2007.00171.x
61. Rosas LG, Eskenazi B. Pesticides and child neurodevelopment. *Curr Opin Pediatr* (2008) **20**(2):191. doi:10.1097/MOP.0b013e3282f60a7d
62. Dietert RR, Dietert JM. Potential for early-life immune insult including developmental immunotoxicity in autism and autism spectrum disorders: focus on critical windows of immune vulnerability. *J Toxicol Environ Health B Crit Rev* (2008) **11**(8):660–80. doi:10.1080/10937400802370923
63. Finegold SM, Molitoris D, Song Y, Liu C, Vaisanen ML, Bolte E, et al. Gastrointestinal microflora studies in late-onset autism. *Clin Infect Dis* (2002) **35**(Suppl 1):S6–16. doi:10.1086/341914
64. Song Y, Liu C, Finegold SM. Real-time PCR quantitation of clostridia in feces of autistic children. *Appl Environ Microbiol* (2004) **70**(11):6459–65. doi:10.1128/AEM.70.11.6459-6465.2004
65. Parracho HM, Bingham MO, Gibson GR, McCartney AL. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *J Med Microbiol* (2005) **54**(10):987–91. doi:10.1099/jmm.0.46101-0
66. Williams BL, Hornig M, Buie T, Bauman ML, Cho Paik M, Wick I, et al. Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. *PLoS One* (2011) **6**(9):e24585. doi:10.1371/journal.pone.0024585
67. Martirosian G, Ekiel A, Aptekorz M, Wiechuła B, Kazek B, Jankowska-Steifer E, et al. Fecal lactoferrin and *Clostridium* spp. in stools of autistic children. *Anaerobe* (2011) **17**(1):43–5. doi:10.1016/j.anaerobe.2010.12.003
68. Bolte ER. Autism and *Clostridium tetani*. *Med Hypotheses* (1998) **51**(2):133–44. doi:10.1016/S0306-9877(98)90107-4
69. Sandler RH, Finegold SM, Bolte ER, Buchanan CP, Maxwell AP, Väisänen ML, et al. Short-term benefit from oral vancomycin treatment of regressive-onset autism. *J Child Neurol* (2000) **15**(7):429–35. doi:10.1177/088307380001500701
70. Noverr MC, Huffnagle GB. Review: the 'microflora hypothesis' of allergic diseases. *Clin Exp Allergy* (2005) **35**(12):1511–20. doi:10.1111/j.1365-2222.2005.02379.x
71. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* (2009) **9**:313–23. doi:10.1038/nri2515
72. Jyonouchi H, Geng L, Streck DL, Toruner GA. Children with autism spectrum disorders (ASD) who exhibit chronic gastrointestinal (GI) symptoms and marked fluctuation of behavioral symptoms exhibit distinct innate immune abnormalities and transcriptional profiles of peripheral blood (PB) monocytes. *J Neuroimmunol* (2011) **238**(1–2):73–80. doi:10.1016/j.jneuroim.2011.07.001
73. Jyonouchi H, Geng L, Ruby A, Reddy C, Zimmerman-Bier B. Evaluation of an association between gastrointestinal symptoms and cytokine production against common dietary proteins in children with autism spectrum disorders. *J Pediatr* (2005) **146**(5):605–10. doi:10.1016/j.jpeds.2005.01.027
74. Jyonouchi H, Geng L, Ruby A, Zimmerman-Bier B. Dysregulated innate immune responses in young children with autism spectrum disorders: their relationship to gastrointestinal symptoms and dietary intervention. *Neuropsychobiology* (2005) **51**(2):77–85. doi:10.1159/000084164
75. Goines PE, Ashwood P. Cytokine dysregulation in autism spectrum disorders (ASD): possible role of the environment. *Neurotoxicol Teratol* (2013) **36**:67–81. doi:10.1016/j.ntt.2012.07.006
76. Unwin LM, Maybery MT, Wray JA, Whitehouse AJO. A "bottom-up" approach to aetiological research in autism spectrum disorders. *Front Hum Neurosci* (2013) **7**:606. doi:10.3389/fnhum.2013.00606
77. Tian Y, Green PG, Stamova B, Hertz-Picciotto I, Pessah IN, Hansen R, et al. Correlations of gene expression with blood lead levels in children with autism compared to typically developing controls. *Neurotox Res* (2011) **19**(1):1–13. doi:10.1007/s12640-009-9126-x
78. Stamova B, Green PG, Tian Y, Hertz-Picciotto I, Pessah IN, Hansen R, et al. Correlations between gene expression and mercury levels in blood of boys with and without autism. *Neurotox Res* (2011) **19**(1):31–48. doi:10.1007/s12640-009-9137-7
79. Kamila M, Markram H. The intense world theory – a unifying theory of the neurobiology of autism. *Front Hum Neurosci* (2010) **4**:224. doi:10.3389/fnhum.2010.00224
80. Reynolds S, Millette A, Devine DP. Sensory and motor characterization in the postnatal valproate rat model of autism. *Dev Neurosci* (2012) **34**:258–67. doi:10.1159/000336646
81. Finegold Sydney M, Downes J, Summanen PH. Microbiology of regressive autism. *Anaerobe* (2012) **18**(2):260–2. doi:10.1016/j.anaerobe.2011.12.018
82. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* (2006) **124**(4):837–48. doi:10.1016/j.cell.2006.02.017
83. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. *PLoS Biol* (2007) **5**:e177. doi:10.1371/journal.pbio.0050177
84. Onore C, Careaga M, Ashwood P. The role of immune dysfunction in the pathophysiology of autism. *Brain Behav Immun* (2012) **26**(3):383–92. doi:10.1016/j.bbi.2011.08.007
85. Schieve LA, Gonzalez V, Boulet SL, Visser SN, Rice CE, Van Naarden Braun K, et al. Concurrent medical conditions and health care use and needs among children with learning and behavioral developmental disabilities, National Health Interview Survey, 2006-2010. *Res Dev Disabil* (2012) **33**(2):467–76. doi:10.1016/j.ridd.2011.10.008
86. Kohane IS, McMurry A, Weber G, MacFadden D, Rappaport L, Kunkel L, et al. The comorbidity burden of children and young adults with autism spectrum disorders. *PLoS One* (2012) **7**(4):e33224. doi:10.1371/journal.pone.0033224
87. Buie T, Fuchs GJ III, Furuta GT, Kooros K, Levy J, Lewis JD, et al. Recommendations for evaluation and treatment of common gastrointestinal problems in children with ASDs. *Pediatrics* (2010) **125**(Suppl 1):S19–29. doi:10.1542/peds.2009-1878D
88. Buie T, Campbell DB, Fuchs GJ III, Furuta GT, Levy J, Van de Water J, et al. Evaluation, diagnosis, and treatment of gastrointestinal disorders in individuals with ASDs: a consensus report. *Pediatrics* (2010) **125**(Suppl 1):S1–18. doi:10.1542/peds.2009-1878C
89. Iwata Y, Tsuchiya KJ, Mikawa S, Nakamura K, Takai Y, Suda S, et al. Serum levels of P-selectin in men with high-functioning autism. *Br J Psychiatry* (2008) **193**:338–9. doi:10.1192/bjp.bp.107.043497
90. Heuer L, Ashwood P, Schauer J, Goines P, Krakowiak P, Hertz-Picciotto I, et al. Reduced levels of immunoglobulin in children with autism correlates with behavioral symptoms. *Autism Res* (2008) **1**:275–83. doi:10.1002/aur.42
91. Grigorenko EL, Han SS, Yrigollen CM, Leng L, Mizue Y, Anderson GM, et al. Macrophage migration inhibitory factor and autism spectrum disorders. *Pediatrics* (2008) **122**:e438–45. doi:10.1542/peds.2007-3604
92. Onore C, Enstrom A, Krakowiak P, Hertz-Picciotto I, Hansen R, Van de Water J, et al. Decreased cellular IL-23 but not IL-17 production in children with autism spectrum disorders. *J Neuroimmunol* (2009) **216**:126–34. doi:10.1016/j.jneuroim.2009.09.005
93. Enstrom AM, Onore CE, Van de Water JA, Ashwood P. Differential monocyte responses to TLR ligands in children with autism spectrum disorders. *Brain Behav Immun* (2010) **24**:64–71. doi:10.1016/j.bbi.2009.08.001
94. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah IN, Van de Water J. Altered T cell responses in children with autism. *Brain Behav Immun* (2010) **25**(5):840–9. doi:10.1016/j.bbi.2010.09.002
95. Goines P, Haapanen L, Boyce R, Duncanson P, Braunschweig D, Delwiche L, et al. Autoantibodies to cerebellum in children with autism associate with behavior. *Brain Behav Immun* (2011) **25**:514–23. doi:10.1016/j.bbi.2010.11.017
96. Kajizuka M, Miyachi T, Matsuzaki H, Iwata K, Shinmura C, Suzuki K, et al. Serum levels of platelet-derived growth factor BB homodimers are increased in male children with autism. *Prog Neuropsychopharmacol Biol Psychiatry* (2010) **34**:154–8. doi:10.1016/j.pnpbp.2009.10.017
97. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah I, Van de Water J. Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain Behav Immun* (2011) **25**:40–45. doi:10.1016/j.bbi.2010.08.003
98. Ross HE, Guo Y, Coleman K, Ousley O, Miller AH. Association of IL-12p70 and IL-6:IL-10 ratio with autism-related behaviors in 22q11.2 deletion syndrome:

a preliminary report. *Brain Behav Immun* (2013) **31**:76–81. doi:10.1016/j.bbi.2012.12.021

Conflict of Interest Statement: The Guest Associate Editor Adam Denes declares that, he holds a permanent position at the Institute of Experimental Medicine, Budapest, Hungary, and despite being a visiting scientist at the same institution as authors Daniel K. Goyal and Jaleel A. Miyan, the review process was handled objectively and no conflict of interest exists.

Received: 23 October 2013; accepted: 20 February 2014; published online: 07 March 2014.

Citation: Goyal DK and Miyan JA (2014) Neuro-immune abnormalities in autism and their relationship with the environment: a variable insult model for autism. Front. Endocrinol. ***5****:29. doi: 10.3389/fendo.2014.00029*

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology.

frontiers in
NEUROSCIENCE

REVIEW ARTICLE
published: 04 March 2014
doi: 10.3389/fnins.2014.00044

Immune mechanisms in cerebral ischemic tolerance

Lidia Garcia-Bonilla, Corinne Benakis, Jamie Moore, Costantino Iadecola and Josef Anrather*

Brain and Mind Research Institute, Weill Cornell Medical College, New York, NY, USA

Edited by:
Adam Denes, University of Manchester, UK

Reviewed by:
Sophie Layé, Université Bordeaux, France
Craig J. Smith, Salford Royal NHS Foundation Trust, UK

****Correspondence:***
Josef Anrather, Brain and Mind Research Institute, Weill Cornell Medical College, 407 East 61st Street, RR-409, New York, NY 10065, USA
e-mail: joa2006@med.cornell.edu

Stressor-induced tolerance is a central mechanism in the response of bacteria, plants, and animals to potentially harmful environmental challenges. This response is characterized by immediate changes in cellular metabolism and by the delayed transcriptional activation or inhibition of genetic programs that are not generally stressor specific (cross-tolerance). These programs are aimed at countering the deleterious effects of the stressor. While induction of this response (preconditioning) can be established at the cellular level, activation of systemic networks is essential for the protection to occur throughout the organs of the body. This is best signified by the phenomenon of remote ischemic preconditioning, whereby application of ischemic stress to one tissue or organ induces ischemic tolerance (IT) in remote organs through humoral, cellular and neural signaling. The immune system is an essential component in cerebral IT acting simultaneously both as mediator and target. This dichotomy is based on the fact that activation of inflammatory pathways is necessary to establish IT and that IT can be, in part, attributed to a subdued immune activation after index ischemia. Here we describe the components of the immune system required for induction of IT and review the mechanisms by which a reprogrammed immune response contributes to the neuroprotection observed after preconditioning. Learning how local and systemic immune factors participate in endogenous neuroprotection could lead to the development of new stroke therapies.

Keywords: preconditioning, ischemic tolerance, stroke, TLR, epigenetics, microRNAs, TNF, inflammation

INTRODUCTION

Inflammation is a central component in the pathophysiology of cerebral ischemia. Brain ischemia triggers both a local and systemic inflammatory response. These responses play contradictory roles: contributing to progression of the ischemic lesion on one hand, and to processes of tissue repair in the injured brain on the other (Iadecola and Anrather, 2011a; Macrez et al., 2011). Since post-ischemic inflammation shows detrimental and beneficial aspects, anti-inflammatory therapies that indiscriminately target both arms of this immune response have not been successful (Iadecola and Anrather, 2011a; Macrez et al., 2011). Therefore, a deeper understanding of post-ischemic inflammation is needed in order to harness its beneficial effects for therapeutic purposes.

In addition to its beneficial role in the repair and regeneration of ischemic tissue, inflammatory pathways are also involved in evoking neuroprotective mechanisms that lead to ischemic tolerance (IT). The brain is equipped with a remarkable capacity to mount self-protective programs that are tuned to limit the deleterious effects of ischemia, commonly referred to as endogenous neuroprotection (Iadecola and Anrather, 2011b). These protective programs can be evoked by preconditioning (PC) stimuli (**Table 1**), such as sublethal stressors, resulting in IT (Kirino, 2002; Narayanan et al., 2013). Thus, understanding the endogenous inflammatory pathways involved in brain IT may lead to novel therapeutic strategies for the prevention and repair of neuronal damage in stroke patients (Shpargel et al., 2008; Macrez et al., 2011).

Cerebral IT can be acquired by several stimuli including short episodes of transient focal and global cerebral ischemia, remote organ ischemia, hypoxia, hypothermia, hyperthermia, exposure to inhalation anesthetics, cortical spreading depression (CSD), brief episodes of seizures or by exposure to low-dose bacterial lipopolysaccharide (LPS) prior to cerebral ischemia (Gidday, 2006; Kunz et al., 2007; Shpargel et al., 2008). IT is characterized by the activation of evolutionary conserved programs that serve to increase the resistance of the brain to ischemia. In the case of early preconditioning, these programs can be activated through preexisting signaling modules that converge in the mitochondria to improved mitochondrial function and energy metabolism during the ischemic event (Dirnagl and Meisel, 2008). In contrast, delayed PC involves gene transcription and protein synthesis (Kitagawa et al., 1990). Because activation of transcription is the main mechanism by which many pro-inflammatory signaling cascades induce a specific cellular response, inflammation is thought to be a significant component of delayed PC. **Table 2** summarizes the inflammatory pathways involved in IT achieved by different PC stimuli (**Table 1**). Inflammation is activated by innate immune receptors such as Toll-like receptors (TLRs) or cytokines receptors such as tumor necrosis factor receptor 1 (TNFR1) and interleukin-1 receptor (IL-1R). Activation of these pathways results in the induction of inflammatory genes that are mediators or effectors of the PC stimulus. In addition to conferring cytoprotection directly, the acquired IT protects the brain by suppressing post-ischemic proinflammatory gene expression,

Table 1 | List of preconditioning stimuli inducing ischemic tolerance.

Abbreviation	Preconditioning stimulus	Achieved by
IPC	Ischemic preconditioning	Transient focal and global cerebral ischemia
RIPC	Remote ischemic preconditioning	Transient occlusion of femoral arteries, mesenteric artery or renal artery
HPC	Hypoxic preconditioning	Exposure to a hypoxic gas mixture
HBO-PC	Hyperoxia or hyperbaric oxygen preconditioning	Exposure to hyperoxia (high oxygen tension) or hyperbaricity (high atmospheric pressure)
Hypo- or hyper-thermic PC	Hypothermic or hyperthermic preconditioning	Decrease or increase of body temperature, respectively
Anesthetic PC	Anesthetic preconditioning	Inhalation of low dose isoflurane or halothane
CSD-PC	Cortical spreading depression preconditioning	Propagation of depolarization wave across the cortical surface
Seizure PC	Seizure preconditioning	Kainic acid injections that induce mild epileptic activity
Exercise PC	Exercise preconditioning	Motor training (treadmill)
TLR-PC	Toll-like receptor preconditioning	Administration of low dose of TLR ligands
LPS-PC	Lipopolysaccharide preconditioning	Administration of low dose of bacterial lipopolysaccharide endotoxin

microglial and endothelial activation and leukocyte infiltration (Huang et al., 2006; Iadecola and Anrather, 2011b).

In this review we will focus on inflammatory pathways leading to cerebral IT. We will review the signaling cascades involved in immune activation in different PC modalities and highlight the molecular components involved. Lastly, this review will highlight the importance of epigenetic modifications and microRNAs in PC-induced reprogramming of the immune system.

EVIDENCE FOR A ROLE OF INFLAMMATORY PATHWAYS IN DIFFERENT PC PARADIGMS

A wide variety of preconditioning stimuli trigger the activation of inflammatory pathways that lead to brain IT (**Table 2**). The receptors, transducers and effector elements of these pathways are shared among different preconditioning paradigms. For example, TLRs are potent mediators of both ischemic (Pradillo et al., 2009; Wang et al., 2010) and endotoxic PC (acquired by systemic administration of LPS) (Tasaki et al., 1997; Ahmed et al., 2000; Vartanian et al., 2011). Likewise, the TNF pathway is involved in ischemic, hypoxic, hyperthermic and exercise-induced PC. Although inflammatory pathways are not independently sufficient for the induction of IT, there is evidence that several PC modalities are dependent upon the induction of such pathways.

ISCHEMIC, HYPOXIC, AND HYPEROXIC PC

Short non-damaging episodes of ischemia prior to a lethal ischemic episode leads to IT (Kirino, 2002). Cerebral IT can be acquired by local PC, mediated either by global (Kitagawa et al., 1990) or focal cerebral ischemia (Barone et al., 1998), or by remote ischemic PC (RIPC), typically achieved by limb ischemia (Tapuria et al., 2008). Although both TLR2 and TLR4 have been implicated in the post-ischemic inflammatory response (Cao et al., 2007; Abe et al., 2010; Brea et al., 2011; Shichita et al., 2012), only TLR4 has been shown to participate in ischemic preconditioning (IPC) (Konstantinov et al., 2004; Pradillo et al., 2009). TLR4 signaling activates transcription nuclear factor-κB (NF-κB) leading to the expression of TNF, inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2), which are required to evoke a PC effect (see **Table 2**). Similarly, TNFR1, another central receptor of the innate immune system and activator of NF-κB, is essential for the induction of IPC (Konstantinov et al., 2004; Pradillo et al., 2005; **Figure 1**).

IT is not limited to the organ to which a stressor is applied. A PC stimulus applied to one organ also leads to IT in other organs, referred to remote PC (for reviews see Tapuria et al., 2008; Anrather and Hallenbeck, 2013; Hess et al., 2013). Remote ischemia can be achieved in rodents by transient occlusion of one or both femoral arteries, the mesenteric artery or the renal artery prior to cerebral ischemia. RIPC results in a decrease of the infarct volume following cerebral ischemia [for a comprehensive list in animal model see (Hess et al., 2013) and (Anrather and Hallenbeck, 2013)]. The effect of RIPC on circulating immune cells has been investigated in humans following forearm transient ischemia (**Table 2**). These studies showed that the expression of pro-inflammatory genes is down-regulated in circulating leukocytes (Konstantinov et al., 2004) and neutrophil function is altered (Shimizu et al., 2010).

Hypoxic-ischemic brain damage is mediated by TLRs (Stridh et al., 2011). Therefore, TLRs might also trigger inflammatory signaling in hypoxic PC (HPC), although no report to date has confirmed their involvement. To induce HPC in rodents, animals are placed in chambers and exposed to a hypoxic gas mixture, usually 8% oxygen and 92% nitrogen, for 3–4 h (Yin et al., 2007; Wacker et al., 2012). Hypoxia-inducible factor 1 (HIF-1) and NF-κB are activated in the brain after HPC (Yin et al., 2007;

Table 2 | Molecular inflammatory mechanisms of brain preconditioning.

PC stimulus	PC type	Receptors	Transducers and effectors induced by PC	References
Ischemia	BCCAo	TLR4	NF-κB, TNF-α, iNOS, COX-2 (48 h)	Pradillo et al., 2009
	MCAo	TNFR1	TACE/TNF-α, NF-κB (48 h)	Pradillo et al., 2005
			TNF-α/TACE (48 h)	Cárdenas et al., 2002
			HO-1, COX-2/PGE_2/ PI3K/Akt (24 h)	Park et al., 2008
			IL-1β gene (6 h), IL-1ra (6 and 24 h)	Shin et al., 2009
			IL-1β (6 h)	Wang et al., 2000
	Remote forearm ischemia	TLR4, TNFR6	HSP70, Calpastatin, TIMP1, ↓caspase-8; PI3KCA, SNAP-23 (24 h)	Konstantinov et al., 2004
			↓neutrophil adhesion and phagocytosis, IL–1β, IL-10 (24 h and 10 days)	Shimizu et al., 2010
			↓platelet activation (PMAs) (5 and 45 min)	Pedersen et al., 2011
	Remote femoral artery ischemia		↑reperfusion (possible protection of endothelium) (48 h)	Vlasov et al., 2005
Hypoxia	8% oxygen, 20 min to 4 h		TNF-α, ceramide (24 h)	Liu et al., 2000
			SphK/S1P (2–4 h), HIF, SphK2, CCL2 (12–24 h)	Wacker et al., 2012
			neuronal CCL2 (12 h), endothelial CCL2 (2 days)	Stowe et al., 2012
			PI3K/Akt/GSK-3β, NF-κB (1–24 h) (post-hypoxic ischemia)	Yin et al., 2007
HBO-PC	Hyperbaric		COX-2 (1–3 days) (post-global ischemia)	Cheng et al., 2011
			HIF-1α, EPO (12 h) (post-MCAo)	Peng et al., 2008
	Normobaric hyperoxia		TNF-α, TACE (24 h)	Bigdeli and Khoshbaten, 2008
Hypothermia	33.0°C, 4 h of reperfusion		↓PMN leucocytes, intercellular adhesion molecule-1 mRNA (4–22 h) (post-MCAo)	Kawai et al., 2000
Hyperthermia	41.5–42°C, 15 min		↑Cortisol (possible prevention of BBB disruption) (24 h)	Ikeda et al., 1999
	38 or 40°C, 6 h		↑HIF-1 alpha expression, HIF-1 binding activity (astrocytes) (0 h)	Du et al., 2010
	42–43°C, 2 h		↑HSP70 (glial, endothelial cells) (24 h)	Ota et al., 2000
Anesthetics	Isoflurane or halothane		↑iNOS (6–24 h)	Kapinya et al., 2002; Zhao and Zuo, 2004
			↑HO-1, NO and TNF-α (macrophages) (24 h)	Li et al., 2009
CSD	0.5 M KCl		↑ERK and COX-2 expression (0–8 h and 2–3 days)	Horiguchi et al., 2005, 2006
	3 M KCl	NMDA receptor	↑TNF-α, IL–1β (4 h)	Jander et al., 2001
Seizure	Kainic acid, bicuculline methiodide, or electrical stimulation		Unknown	Vezzani et al., 2002

(Continued)

Table 2 | Continued

PC stimulus	PC type	Receptors	Transducers and effectors induced by PC	References
Exercise	Treadmill		↑TNF-α (0 h)	Ding et al., 2005
TLR ligands	LPS	TLR4	NF-κB suppression; IRF3, Ship1, Tollip, p105 (post-MCAo) (72 h)	Sly et al., 2004; Vartanian et al., 2011
			Genes related to TLR pathway and cytokine–cytokine receptor interaction pathway (3 h)	Marsh et al., 2009b; Stevens et al., 2011
			IRF3, IFNβ (3 and 24 h)	Marsh et al., 2009b
			↑Ceramide (6–12 and 48 h)	Zimmermann et al., 2001
			↑PMN infiltration (post-MCAo) (6 and 24 h)	Ahmed et al., 2000
	CpG ODN	TLR9	IRF3, IRF7, type I IFN gene expression (post-MCAo) (3 and 24 h); TNF-α (serum) (1 h)	Stevens et al., 2008, 2011
	GDQ	TLR7, IFNAR	IRF7, IFNα (1–2 h)	Leung et al., 2012
	Poly-ICLC	TLR3	↑plasma levels of IL-1β, IL-6, IL-12, TNF-α, and IFNγ compared to LPS-PC (3 h)	Packard et al., 2012
			↑IFN-β (protein 6–8 h and mRNA 6–24 h), preservation of BBB endothelial cell	Gesuete et al., 2012
	Pam3CSK4	TLR2	↑zonula occludens-1 (ZO-1), no loss of occludin protein inducing preservation of BBB (6 and 24 h)	Hua et al., 2008

A PC stimulus (column 1) may be divided in several PC types (column 2) that through the stimulation of several receptors (column 3), activate different transducers and effectors of inflammatory pathways (column 4), accounting for the protective mechanism of the brain. Time points in brackets indicate the time point of analysis relative to the PC induction; post-MCAo indicates that the analysis was performed after induction of index ischemia.

ATA, atmosphere abolute pressure; BBB, Blood Brain Barrier; BCCAo, Bilateral Common Carotid Artery Occlusion; CCL2, chemokine (C-C motif) ligand; CCR1, 5, Chemokine Receptor 1, 5; COX-2, Cyclooxygenase 2; CpG ODN, cytosine-guanine oligodeoxynucleotides; CSD, Cortical Spreading Depression, EPO, Erythropoietin; GDQ, Gardiquimod; GSK-3β, Glycogen Synthase Kinase 3; HBO-PC, Hyperbaric Oxygen Preconditioning; HIF, Hypoxia-Inducible Factor; HO-1, Heme Oxygenase-1; HSP70, Heat Shock Protein 70; IFN, Interferon; IFNAR, type I interferon receptor; IL-1β, Interleukin 1β; IL-1ra, IL-1 receptor antagonist; iNOS, Inducible Nitric Oxidase Synthase; IPC, Ischemic Preconditioning; IRF, Interferon Regulatory Factor; KCl, Chloride potassium; LPS, lipopolysaccharide; MCAo, Middle Cerebral Artery occlusion; MCPIP1, Monocyte Chemotactic Protein–Induced Protein 1; MyD88, Myeloid differentiation primary response 88; NF-κB, Nuclear Factor-κB; NMDA, N-methyl-D-aspartate; NO, Nitric Oxide; ODN, oligodeoxynucleotide; Pam3CSL4, Pam3CysSerLys4; PGE_2, Prostaglandin E2; PI3K, Phosphatidylinositol 3-Kinase; PI3KCA-γ, PI3K catalytic subunit gamma isoform; PMA, platelet–monocyte aggregates; PMN, polymorphonuclear; Poly-ICLC, polyinosinic polycytidylic acid; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; S1P, Sphingosine-1-phosphate; Ship1, Src homology-2 domain-containing inositol 5-phosphatase 1; SNAP-23, Synaptosome-associated protein-23; SphK2, Sphingosine Kinase 2; TACE, Tumor Necrosis factor-alpha-Converting Enzyme; TIMP1, Tissue inhibitor of metalloproteinase 1; TLR, Toll-Like Receptor; TNFR, Tumor necrosis factor receptor; TNF-α, Tumor necrosis factor alpha; Tollip, toll interacting protein; TRIF, TIR-domain-containing adapter-inducing interferon-β.

Wacker et al., 2012) leading to the expression of iNOS (Jung et al., 2000), TNF-α (Liu et al., 2000) and chemokine (C-C motif) ligand 2 (CCL2) (Stowe et al., 2012), which have been implicated in establishing IT after HPC (see below section Molecular Components of the Immune System Involved in PC).

The immune system is also involved in hyperoxic PC, which can be achieved by either normobaric hyperoxia or hyperbaric oxygen PC (HBO-PC). Delivering a gas mixture containing 90–95% oxygen induces normobaric hyperoxia. HBO-PC consists of the inspiration of an oxygen-containing gas mixture at higher than one atmospheric absolute pressure (ATA), usually 2–2.5 ATA. Bigdeli et al. showed that rats subjected to 16 h of normobaric hyperoxia had reduced infarct volume after Middle Cerebral Artery occlusion (MCAo) and that the TNF-α converting enzyme (TACE)/TNF-α/NF-κB pathway is associated with the protective effect (Bigdeli and Khoshbaten, 2008). Moreover, COX-2 has also been reported to be a mediator of HBO-PC (Cheng et al., 2011). Administration of the specific COX-2 inhibitor, NS-398, before HBO exposure reversed the protective effect of HBO-PC. Cheng et al. further identified COX-2-dependent upregulation of HIF-1α as a possible down-stream mediator of HBO-PC.

HYPOTHERMIC AND HYPERTHERMIC PC

Hypothermia induced during and/or after ischemia has a well-established protective effect in acute ischemic stroke (Wu and Grotta, 2013) and may be partly due the inhibition of the inflammatory response occurring during stroke (Ceulemans et al.,

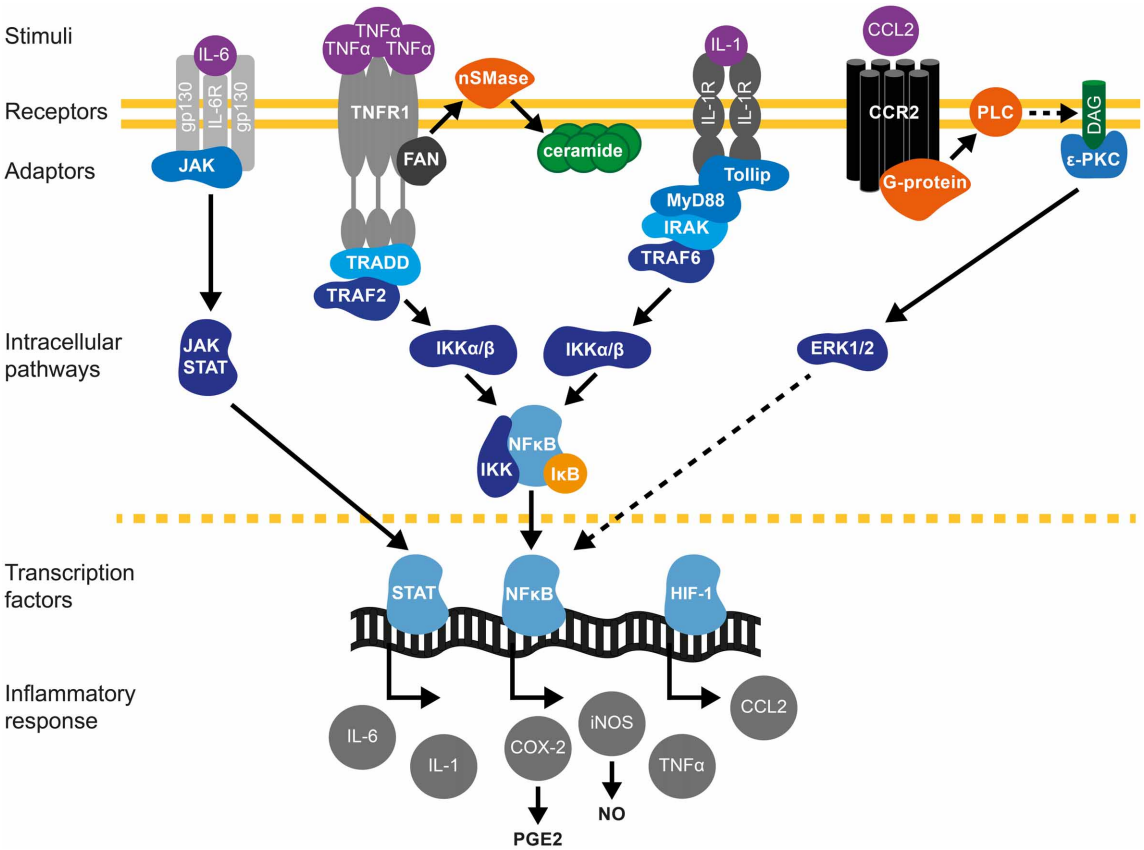


FIGURE 1 | Cytokine signaling pathways in brain preconditioning. Following preconditioning (PC), the increase in tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 and chemokine (C-C motif) ligand 2 (CCL2) leads to the activation of inflammatory cascades and induction of gene expression such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, mediators of delayed PC. IL-6/IL-6R activates Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. Phosphorylation and nuclear translocation of STAT induces iNOS and COX-2 up-regulation (Dawn et al., 2004). TNF-α activates TNF receptor 1(TNFR1)/Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD)/TNF-α receptor–associated factor 2 (TRAF2) pathway (Hallenbeck, 2002; Pradillo et al., 2005). Furthermore, TNF-α activation promotes ceramide synthesis that also mediates TNFα-preconditioning (Liu et al., 2000; Ginis et al., 2002). The neutral sphingomyelinase domain (NSD) of TNFR1 recruits factor associated with neutral sphingomyelinase activation (FAN). FAN activates neutral sphingomyelinase (nSMase) in the outer leaflet of the plasma membrane to produce ceramide (Hallenbeck, 2002). IL-1R activates toll interacting protein (tollip)/myeloid differentiation primary response 88 (MyD88)-IL-1R-associated kinase (IRAK)/TRAF6 signaling. TRAF mediates the activation of inhibitor of κB kinase (IKK), which triggers the phosphorylation-induced proteasomal degradation of the IκB, NF-κB activation and subsequent iNOS and COX-2 expression. CCL2/CCR2 activation promotes phospholipase-C (PLC)-diacylglycerol (DAG)-protein kinase C (PKC) pathway that leads to extracellular signal-regulated kinases (ERK1/2) activation and ultimately causes NF-κB activation (Rehni and Singh, 2012). In addition, HIF-1-induced expression of CCL-2 confers cerebral ischemic tolerance (Stowe et al., 2012). COX-2-derived prostaglandin E2 (PGE_2) (Horiguchi et al., 2006) and iNOS-derived nitric oxide (NO) (Cho et al., 2005) are molecular mediators of PC.

2010). Brief exposure to hyperthermia represents an additional method of PC (**Table 2**). In animal model of stroke, transient hypothermia prior an ischemic insult (Nishio et al., 2000; Yunoki et al., 2003) or during reperfusion (Kawai et al., 2000) reduces infarct volume. The latter study has reported a decrease number of polymorphonuclear leucocytes during reperfusion—shown by a reduced myeloperoxidase activity in rats subjected to hypothermia (Kawai et al., 2000). Hyperthermic PC reduces brain damage in newborn rats as well (Ota et al., 2000). The protective mechanism of hyperthermic PC has been linked to the induction of the heat shock protein 70 (HSP70) within glial and endothelial cells (Ota et al., 2000). In addition to its role as an intracellular chaperon, HSP70 acts as an endogenous ligand of TLR4. Extracellular HSP70 is released from damaged cells and serves as a damage-associated molecular pattern (DAMPs) molecule (Bianchi, 2007). It has been shown that HSP70 mediates endotoxin tolerance by signaling through the TLR4/CD14 pathway (Vabulas et al., 2002; Aneja et al., 2006), similar to the canonical TLR4 ligand LPS. Thus, induction of HSP70 following hyperthermia could be a factor in the establishment of IT.

ANESTHETIC PC

Inhalation anesthetics are strong inducers of IT and can directly modulate immune functions in neutrophils, monocytes and lymphocytes (Clarkson, 2007; Wang et al., 2008). Rats pretreated with low dose isoflurane or halothane were protected after permanent MCAo (Kapinya et al., 2002). The protective effect was attributed to increased iNOS expression after anesthetic PC, and

was reversed by treating the animals with the iNOS inhibitor aminoguanidine. Volatile anesthetics might directly alter the response of immune cells to inflammatory stimuli. Isoflurane PC of mouse macrophages induced the expression of heme oxygenase-1 (HO-1) and decreased NO and TNF-α release after subsequent LPS exposure (Li et al., 2009).

CORTICAL SPREADING DEPRESSION, BRAIN STIMULATION, AND SEIZURES

CSD is an electrophysiological phenomenon characterized by a slowly propagating depolarization wave across the cortical surface that confers delayed IT (Yanamoto et al., 1998; Horiguchi et al., 2006; Shpargel et al., 2008). In animal models, CSD PC is elicited by either topical application or superfusion of the cortical surface with a 0.5–5 M KCl solution that induces IT lasting 1–15 days (Kawahara et al., 1995; Yanamoto et al., 1998; Horiguchi et al., 2006). The delayed tolerance induced by CSD is protein synthesis dependent and has been linked to the up-regulation of trophic factors and glial cell activation (Kawahara et al., 1999). On the other hand, spreading depression in organotypic hippocampal slice cultures increases the expression of several pro-inflammatory cytokines including TNF-α, IL-1β, IL-1α, and IL-6 (Kunkler et al., 2004). Indeed, *in vivo* experiments found an early induction of both TNF-α and IL-1β after CSD (Jander et al., 2001). Pre-treatment with the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 completely abolished the upregulation of these cytokines, implicating NMDA receptors as a critical element driving their production. Additionally, Horiguchi et al. reported that CSD-induced neuroprotection against ischemic injury resulting from MCAo is dependent upon increased COX-2 expression (Horiguchi et al., 2005, 2006), supporting the involvement of the inflammatory response in CSD PC.

Electrical stimulation of the cerebellar fastigial nucleus, but not other cerebellar nuclei, can induce potent and long-lasting protection from focal or global cerebral ischemic injury (Reis et al., 1991, 1998; Golanov et al., 1998). At the same time, fastigial nucleus stimulation evokes a strong anti-inflammatory response and suppresses post-ischemic iNOS expression and brain inflammation after cortical IL-1β injection (Galea et al., 1998a,b). The effect is mediated, at least in part, by increasing the tolerance of mitochondria to calcium overload, and suppressing the release of mitochondrial pro-apoptotic factors induced by cerebral ischemia (Zhou et al., 2005). The molecular mechanisms of the protective effects on mitochondria involve upregulation of prohibitin, an integral protein of the inner mitochondrial membrane, which protects mitochondrial structure and function during cell stress (Zhou et al., 2012). Consistent with its role in PC, overexpression of prohibitin renders neurons more resistant to injury in a wide variety of models (Zhou et al., 2012).

Neuroprotection against cerebral damage induced by lethal ischemic/hypoxia or global ischemia can also be acquired through induction of mild epileptic activity elicited by kainic acid injections (Plamondon et al., 1999; Towfighi et al., 1999). Although the mechanisms are unknown, synthesis and release of TNF-α, IL-1β, IL-1α, and IL-6 by glia might contribute to this tolerance modality (Vezzani et al., 2002).

EXERCISE PC

Physical exercise prevents stroke and forced exercise training affords neuroprotection against ischemic injury (Endres et al., 2003). Experimentally, exercise PC can be achieved by training rodents on a motor driven treadmill for 1–3 weeks (Wang et al., 2001; Ding et al., 2005; Curry et al., 2010). Among other mechanisms, such as protection against the blood-brain barrier (BBB) disruption, promotion of angiogenesis and inhibition of apoptosis, exercise PC involves activation of the immune system (Zhang et al., 2011). The skeletal muscle is an important source of several cytokines, often referred to as myokines, including IL-6, IL-8, IL-15, BDNF, LIF, and FGF21 (Pedersen, 2011). The release of these myokines into circulation might be responsible for the systemic effects of exercise, including its neuroprotective potential (Iadecola and Anrather, 2011b). Downstream mediators of exercise PC may include TNF-α (Ding et al., 2005) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Guo et al., 2008; Curry et al., 2010). In one study, exercised rats submitted to MCAo showed reduced infarct volume when compared to non-exercised rats and this protection was associated with a gradual increase in the level of TNF-α in the brain over the 3 week course of treadmill exercise (Ding et al., 2005). Pre-ischemic blockage of TNF-α signaling with an anti-TNF-α antibody or inhibition of ERK1/2 activation abolished the protective effect (Guo et al., 2008). Exercise PC can also change the expression of innate immunity receptors. Treadmill exercise decreased cerebral TLR4 receptor expression in rats, although the importance of this finding as a mechanism for cerebral IT has not been investigated to date (Zwagerman et al., 2010).

MOLECULAR COMPONENTS OF THE IMMUNE SYSTEM INVOLVED IN PC

Inflammatory signaling is governed by a complex array of molecules that not only modulate the response of the target cell, but also change the local microenvironment and cellular tissue composition by recruiting cell types that are not normally present in that tissue. This inflammatory cascade is driven by cytokines, chemokines and their receptors and is regulated by immunomodulatory molecules. Several of these components have been implicated in PC. Evidence for direct involvement of some of these immune factors in cerebral IT is summarized in **Table 3**.

TLRs

The Toll receptor was first described in the context of Drosophila embryogenesis (Anderson et al., 1985). Subsequently, it was discovered that the Toll pathway is activated upon Drosophila fungal infection revealing a role of Toll receptors in the immune response (Lemaitre et al., 1996). Exposure of immune cells to certain pathogens activates these receptors resulting, in the activation of intracellular signaling pathways that lead to the expression of genes involved in the inflammatory response. In vertebrates, TLRs, named for their homology to the *toll* gene in Drosophila, are transmembrane proteins containing domains that are able to recognize viral or microbial components known as pathogen-associated molecular patterns (PAMPs) (Wang et al., 2011). Peptidoglycans (PGN), lipoproteins, lipoteichoic acids (LTA),

Table 3 | Evidence for the involvement of inflammatory mediators in PC.

Inflammatory mediators	Deletion/ Inhibitor	PC type (dose)/time of application prior to brain ischemia	Index ischemia	Species	Outcome	References
Chemokine receptors	CCL2 KO	HPC 2 days prior	tMCAo	Mouse	Reversed	Stowe et al., 2012
	CCL2 antibody	HPC 2 days prior	tMCAo	Mouse	Reversed	Stowe et al., 2012
Cytokines recpetors	TNF-α KO	LPS (0.2 mg/Kg) 3 days prior	tMCAo	Mouse	Reversed	Rosenzweig et al., 2007
	TNF-α KO	CpG ODN (1.6 mg/Kg) 3 days prior	tMCAo	Mouse	Reversed	Stevens et al., 2008
	TNFbp	LPS (0.2 mg/Kg) 2–4 days prior	pMCAo	SHR	Reversed	Tasaki et al., 1997
	IL-1ra	BCCo 3 days prior	BCCAo	Gerbil	Reversed	Ohtsuki et al., 1996
TLR receptors	TLR2 KO	Pam3CSK4 1 h prior	tMCAo	Mouse	Reversed	Lu et al., 2011
	TLR4 KO	BCCAo 2 days prior	pMCAo	Mouse	Partially reversed	Pradillo et al., 2009
	TLR7 KO	GDQ (40 μg/mouse) 3 days prior	tMCAo	Mouse	Reversed	Leung et al., 2009
TLRs adaptors	TRIF KO	LPS (0.4 mg/Kg) 3 days prior	tMCAo	Mouse	Reversed	Vartanian et al., 2011
	IRF3 KO	LPS (0.5 mg/Kg) 3 days prior	tMCAo	Mouse	Reversed	Marsh et al., 2009b
	IRF3 KO	CpG ODN (1.6 mg/Kg) 3 days prior	tMCAo	Mouse	Reversed	Stevens et al., 2011
	IRF3 KO	tMCAo 3 days prior	tMCAo	Mouse	Partially reversed	Stevens et al., 2011
	IRF7 KO	LPS (1 mg/Kg) 3 days prior	tMCAo	Mouse	Reversed	Stevens et al., 2011
	IRF7 KO	CpG ODN (1.6 mg/Kg) 3 days prior	tMCAo	Mouse	Reversed	Stevens et al., 2011
	IRF7 KO	tMCAo 3 days prior	tMCAo	Mouse	Partially reversed	Stevens et al., 2011
iNOS	iNOS KO	tMCAo 1 day prior	tMCAo	Mouse	Reversed	Cho et al., 2005
	Amino- guanidine (iNOS)	tMCAO 1 day prior	tMCAo	Mouse	Reversed	Cho et al., 2005
	Amino- guanidine (iNOS)	LPS (0.5 mg/Kg) 1 day prior	tMCAo	Mouse	Reversed	Cho et al., 2005
	Amino- guanidine (iNOS)	Isoflurane 1 day prior	pMCAo	Rat	Reversed	Kapinya et al., 2002
COX-2	NS-398 (COX-2)	HBO 5 days prior	4VO	Rat	Reversed	Cheng et al., 2011
	Rofecoxib (COX-2)	tMCAO 8 h prior	pMCAo	Rat	Reversed	Park et al., 2008
NF-κB pathway	DTTC (NF-κB)	4VO 3 days prior	4VO	Rat	Reversed	Blondeau et al., 2001
	κB decoy DNA (NF-κB)	4VO 3 days prior	4VO	Rat	Reversed	Blondeau et al., 2001
	DTTC (NF-κB)	linolenic acid (500 nmol/Kg) 3 days prior	4VO	Rat	Reversed	Blondeau et al., 2001
	κB decoy DNA (NF-κB)	linolenic acid (500 nmol/Kg) 3 days prior	4VO	Rat	Reversed	Blondeau et al., 2001

The deletion or inhibition of chemokines, cytokines, or TLR receptors, iNOS and COX-2 molecules and TLR or NF-κB signaling components described in the table, are able to abolish the preconditioning effect, unveiling the role of inflammatory pathways involved in IT.

4VO, 4 Vessel Occlusion; BCCAo, Bilateral Common Carotid Artery occlusion; COX-2, cyclooxygenase-2; CpG ODN, cytosine-guanine oligodeoxynucleotides; DTTC, diethyldithiocarbamate; GDQ, Gardiquimod; HBO-PC, Hyperbaric Oxygen Preconditioning; HPC, hypoxic preconditioning; IL-1ra, Interleukin 1 Receptor Antagonist; iNOS, inducible Nitric Oxide Synthase; IRF, Interferon (IFN)-regulatory factor; LPS, Lipopolysaccharide; pMCAo, permanent Middle Cerebral Artery occlusion; TLR, Toll-Like Receptor; SHR, Spontaneously Hypertensive Rats; tMCAo, transient Middle Cerebral Artery occlusion; TNFbp, Tumor Necrosis Factor binding protein; TNF-α, Tumor Necrosis Factor α; TRIF, TIR-domain-containing adapter-inducing interferon-β.

lipopolysaccharides (LPS), as well as viral and bacterial nucleic acids, serve as PAMPs recognized by individual TLRs (Akira, 2009). To date 13 members of the TLR family have been identified in mammals. Among the most extensively studied TLRs are TLR2 which binds to LTA component of Gram-positive bacteria and TLR4, which recognizes LPS on the cell wall of Gram-negative bacteria (Verstak et al., 2007). Upon ligand binding, TLRs further signal by recruiting intracellular Toll/IL-1R (TIR)-homology

domain containing adaptor proteins, which selectively activate signaling cascades that lead to immune responses. Several TLR adapter molecules that are associated with functionally different signaling cascades, such as myeloid differentiation factor-88 (MyD88) and TIR domain containing adaptor protein inducing interferon β (TRIF), are known to date. With the exception of TLR3, TLRs signal through MyD88-dependent pathways, while TLR4 can activate both MyD88-dependent and—independent pathways (Wang et al., 2011; Mallard, 2012).

MyD88 mobilizes members of the IL-1R-associated kinase family (IRAK) leading to nuclear translocation of the transcription factor NF-κB. NF-κB is a major regulator of the inflammatory response (Li and Verma, 2002) and is responsible for the induction of various inflammatory genes including cytokines, chemokines, adhesion molecules, proinflammatory enzymes, and growth factors (Pahl, 1999). NF-κB is commonly found as a heterodimer consisting of p50 (NFKB1) and p65 (RelA) subunits. In resting cells, the inactive form of NF-κB is located in the cytoplasm by association with inhibitor proteins, IκBα and IκBβ (Baeuerle and Henkel, 1994). Upon stimulation, IκB proteins are phosphorylated and proteolytically degraded, and NF-κB dimers are able to translocate to the nucleus and bind to the promoter region of target genes initiating their transcription (Baldwin, 1996). NF-κB is activated in many cell types including cells of the central nervous system (Kaltschmidt et al., 1994). In the rodent brain, NF-κB can be activated by numerous factors known to be induced after ischemia-reperfusion, such as glutamate, increased intracellular Ca^{2+}, reactive oxygen species (ROS) and inflammatory cytokines (Harari and Liao, 2010). Furthermore, target genes of NF-κB have been implicated in the pathogenesis of cerebral ischemia, such as IL-1, IL-6, TNF-α, ICAM-1, MMP9, COX-2, and iNOS (Baeuerle and Henkel, 1994; Allan et al., 2005; Kaltschmidt et al., 2005; Harari and Liao, 2010). Thus, it has been postulated that stimulation of signaling pathways that lead to NF-κB activation are associated with detrimental outcome in cerebral ischemia (Stephenson et al., 2000). Supporting this notion, inhibition of NF-κB using pharmacologic agents results in reduced infarct size after stroke in rodents (Clemens et al., 1998; Nurmi et al., 2004). It has also been shown that mice lacking the p50 NF-κB subunit have reduced ischemic damage (Schwaninger et al., 1999; Nurmi, 2004). However, subsequent studies have also revealed a deleterious effect of NF-κB inhibition in neonatal hypoxia (Nijboer et al., 2008) and an increase of neuronal death after transient ischemia in p50 deficient mice (Duckworth et al., 2006) raising the possibility for a dual role of NF-κB in stroke.

TLR4 stimulation also leads to the activation of MyD88-independent pathways. In this case, binding of the adaptor molecule TRIF activates the transcription factors interferon regulatory factor 3 (IRF3) and 7 (IRF7), resulting in expression of IFNα and IFNβ type I IFN genes (Marsh et al., 2009a; Wang et al., 2011; Mallard, 2012) (**Figure 2**). Type I IFNs are released in the extracellular space and signal in an auto- or paracrine manner through a single heterodimeric receptor, IFNAR, to activate the JAK/STAT pathway, leading to the expression of several chemokines such as CCL2, CCL7, and CXCL10, while inhibiting CXCL1 and CXCL2 expression. Depending on the concentration of IFN and type of target cell, the resulting immune response can be pro- or anti-inflammatory (Trinchieri, 2010).

Aside from binding to PAMPs, TLRs also recognize endogenous molecules known as DAMPs, which are released during ischemic cellular injury (Chen and Nuñez, 2010). Prototypical DAMPs released from injured cells include high-mobility group box 1 (HMGb1) protein that activates TLR2, 4, and 9; heat shock proteins (HSPs; TLR2, 4); RNA (TLR3); mitochondrial DNA (TLR9); hyaluronic acid (TLR2, TLR4) and peroxiredoxins (TLR2, TLR4) (Chen and Nuñez, 2010; Patel et al., 2012; Shichita et al., 2012). HMGb1 and peroxiredoxins have been reported to increase cerebral ischemic injury by augmenting the post-ischemic inflammatory response (Kim, 2006; Yang et al., 2011; Shichita et al., 2012). While the role of DAMPs in cerebral IT has not been addressed, it has been shown that systemic administration of HMGb1 protects against myocardial ischemia–reperfusion injury and liver ischemia-reperfusion injury, a process that involves TLR4 (Izuishi et al., 2006; Hu et al., 2010). Additionally, HSP70 mediates endotoxin tolerance by signaling through TLR4 receptors (Aneja et al., 2006). Therefore, these findings support DAMPs to participate in the PC effect through TLRs activation. In contrast to the detrimental effect of TLR activation in response to ischemia (Wang et al., 2011), stimulation of some TLRs prior to ischemia provides robust neuroprotection (Marsh et al., 2009a; Wang et al., 2011). The ability of TLR ligands to induce cerebral IT was first demonstrated after systemic administration of low-dose LPS, the major TLR4 ligand, causing spontaneously hypertensive rats to become tolerant to subsequent ischemic brain damage induced by MCAo (Tasaki et al., 1997). Subsequently, LPS-induced tolerance to brain ischemia has been demonstrated in several experimental stroke models in mice, gerbils and pigs (Rosenzweig et al., 2004; Hickey et al., 2007; Yu et al., 2010). A dose below 1 mg/Kg of LPS administered 1–3 days prior to cerebral ischemia is generally used in rodents to induce IT. Furthermore, TLR4-mutant mice were refractory to IPC, indicating that TLR4 plays an universal role in establishing IT (Pradillo et al., 2009). Comparable to TLR4, pre-treatment with the TLR2 lipopetide ligand Pam3CysSerLys4 (Pam3CSK4) resulted in significantly decreased cerebral infarct volume after focal cerebral ischemia/reperfusion in mice (Hua et al., 2008). Preservation of the BBB has been suggested to play a role in this PC mechanism. Similarly, stimulation of TLR7 by administration of its agonist gardiquimod, an imidazoquinoline compound, provides neuroprotection against ischemic injury and was associated with the up-regulation of the IFN pathway and IFNα production (Leung et al., 2012). Interestingly and in contrast to TLR4 and TLR9 induced PC, TLR7-mediated PC was abolished in IFNAR mutant mice. Finally, activation of TLR9 by unmethylated CpG oligodeoxynucleotides induced IT after transient MCAo in mice (Stevens et al., 2008). Similar to LPS-PC, the protective effect after TLR9 stimulation lasted for up to a week and was dependent on the expression of TNF-α (**Figure 2A**) (Stevens et al., 2008; Marsh et al., 2009a). Accordingly, CpG oligodeoxynucleotides administration to TNF-α null mice failed to induce IT.

IT induced by TLR activation involves the reprogramming of inflammatory pathways. This is achieved by at least two different mechanisms. Concomitantly with induction of pro-inflammatory

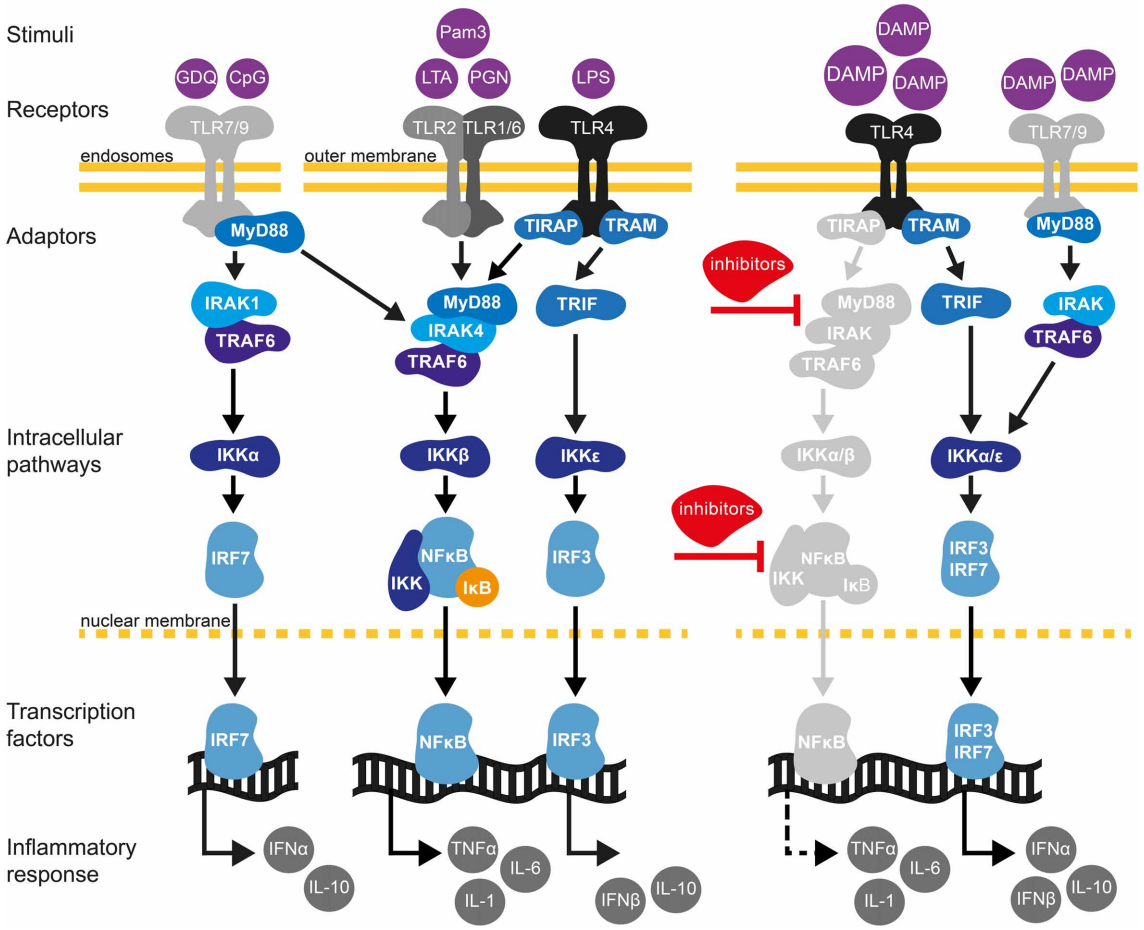


FIGURE 2 | TLR signaling pathways in cerebral IT. (A) Preconditioning through TLRs may be afford by exposure with low dose of pathogen-associated molecular pattern (PAMPs) molecules (Mallard, 2012). TLRs are localized either at the outer cell surface (TLR1, TLR2, TLR4, and TLR6) or within endosomes (TLR7 and TLR9) (Marsh et al., 2009a). TLR2 dimerizes with TLR1 or TLR6 and is activated upon binding of PAMPs such as the synthetic lipopeptide Pam3CysSerLys4 (Pam3CSK4), the non-peptide ligand lipoteichoic acid (LTA) or peptidoglycans (PGN). TLR4 predominantly recognizes lipopolysaccharides (LPS) from Gram-negative bacteria. TLR7 and TRL9 can be activated by the synthetic imidazoquinoline Gardiquimod (GDQ) or small DNA such as non-methylated cytosine-guanosine (CpG), respectively (Wang et al., 2011). Preconditioning of TLRs will favor the activation of the myeloid differentiation factor-88 (MyD88)-dependent pathway. Upon binding of ligands, MyD88 is activated either directly (TLR7/9) or via the toll-interleukin 1 receptor (TIR) domain containing adaptor protein TIRAP (TLR2/4). The latter further mobilizes members of the IL-1R-associated kinase family (IRAK) leading to the subsequent binding/activation of TNF receptor associated factor-6 (TRAF6), the inhibitor of κB kinase (IKK) complex (composed of IKKα and IKKβ) and IκB. Once IκB is phosphorylated and degraded by the proteasome, nuclear factor-κB (NF-κB) translocates to the nucleus leading to low level of pro-inflammatory cytokine synthesis, such as TNF-α. TLR PC also induces the production of several negative inhibitors of TLR signaling, targeting mainly MyD88 and NF-κB pathways. Induction of proinflammatory cytokines and activation of such inhibitors are required to induce a state of ischemic tolerance (Marsh et al., 2009a; Vartanian and Stenzel-Poore, 2010; Wang et al., 2011). In addition to activating the MyD88-dependent pathway, TLR4 signals through the MyD88-independent pathway to activate IRF3 by sequential recruiting of the adaptor molecule TIR inducing interferon β (TRIF) and IKKε resulting in expression of anti-inflammatory type I interferon (IFN) genes, including IFNβ (Marsh et al., 2009b). Type I IFNγ genes are also induced by TLR7/9 activation through the TRAF6/IKKα pathway (Hoshino et al., 2006). **(B)** Damage-associated molecular pattern (DAMPs) molecules are endogenous ligands of TLRs produced in response to ischemic injury (Vabulas et al., 2002). Following preconditioning, activation of TLR4/7/9 by DAMPs will fail to activate MyD88 and the subsequent signaling molecules, such as IRAK and NF-κB. Indeed, several inhibitors produced during the first exposition of TLRs to exogenous stimuli will block the signaling of TLRs to NF-κB. Whether pro-inflammatory cytokines are suppressed or up-regulated is controversial and might be model specific (Marsh et al., 2009a; Vartanian and Stenzel-Poore, 2010). On one hand, TLR preconditioning induces a down-regulation of NF-κB. On the other hand, it has been shown that neuroprotection following cerebral ischemia is achieved by an up-regulation of the TLR-IRF axis generating an increase of TGF-β, IL-10, IFNα, and IFNβ anti-inflammatory cytokines (Stevens et al., 2011; Leung et al., 2012). Thus, TLR preconditioning induces a reorganization of the TLR signaling pathways after cerebral ischemia toward TRIF/IRF signaling that may confer protection of the brain against ischemic damage.

mediators, activation of the MyD88-dependent pathway will result in the upregulation of negative regulators, which prevent the interaction of several adaptors along the TLR4-MyD88 pathway (**Figure 2A**) (Sly et al., 2004). At the same time, activating the MyD88-independent pathway evokes a type I IFN response. Indeed, recent studies have found a neuroprotective role of the MyD88-independent pathway in TLR-induced IT after stroke (Marsh et al., 2009a). It has been postulated that pretreatment with LPS induces a switch in the transcriptional response to subsequent TLR4 stimulation by increasing the expression of the IRF3-induced cytokine IFNβ (Veldhuis et al., 2003) (**Figure 2B**). Consistent with this hypothesis, gene expression analysis following LPS-PC revealed upregulation of inhibitors of the Myd88-dependent and NF-κB pathways (Vartanian et al., 2011). Interestingly, the expression of pro-inflammatory genes related to the NF-κB signaling pathway was similar in both mice undergoing LPS-PC and in control mice following cerebral ischemia (**Figure 2B**). However, the study showed that LPS-PC increased IRF3 activity following ischemia and evoked the upregulation of anti-inflammatory genes including TGF-β and IL-10 (Vartanian et al., 2011). The importance of the MyD88-independent pathway in establishing IT is further supported by the fact that LPS-PC was unable to induce IT in TRIF- but not in Myd88-deficient mice (Vartanian et al., 2011).

CYTOKINES

Cytokines are secreted proteins with growth, differentiation, and activation functions that shape the nature of the immune response. Among the more than 70 candidate cytokines, TNF-α, IL-1, IL-6, and IL-17 play major roles in initiating and amplifying the post-ischemic inflammatory response, while IL-10 and TGF-β are the main anti-inflammatory factors (Iadecola and Anrather, 2011a). Since immune cells, including monocytes/macrophages, in the periphery and microglia in the brain express TLR receptors (Downes and Crack, 2010), they are capable of responding to systemic TLR agonists used as a preconditioning stimulus and modify cytokine expression or secretion (Rosenzweig et al., 2004; Ransohoff and Brown, 2012). Accordingly, several cytokines secreted by cells of the innate immune system have been implicated in PC. For example, TNF-α has been identified as an essential mediator of LPS-PC. It has been shown that LPS-preconditioned TNF-α null mice were not protected from ischemic brain injury (Rosenzweig et al., 2007). This study confirmed previous findings demonstrating that administration of a specific TNF antagonist reversed the protective effect of LPS PC in a model of permanent focal ischemia in mice (Tasaki et al., 1997). Similarly, upregulation of IL-1α and IL-1β after bilateral common carotid artery occlusion (BCCAo) triggered tolerance to global ischemia (Ohtsuki et al., 1996; Shin et al., 2009). Ohtsuki et al. also showed that systemic delivery of either IL-1α or IL-1β was sufficient to induce IT to a subsequent episode of lethal global ischemia and that intraperitoneal injection of recombinant human IL-1 receptor antagonist prior to IPC abolished the neuroprotective effect.

In addition to systemic administration of cytokines, neuroprotection has also been reported with direct delivery of cytokines to the brain before ischemia. Previous intracerebrovascular injection of IL-6 in rats subjected to permanent ischemia protects from cell death (Loddick et al., 1998). Likewise, intracisternal administration of TNF-α in mice before distal MCAo is neuroprotective (Nawashiro et al., 1997). However, in the same study, both intravenous and intraperitoneal routes of TNF-α administration failed to exert neuroprotection against cerebral ischemia. While differences in modality of administration and stroke models may account for the observed disparities in outcome, it remains unclear how systemic cytokines exert their effect within the brain. TNF-α, IL-1β, and IL-6 are transported unidirectionally across the BBB (Banks, 2005). Thus, systemic induction of cytokines can lead to an increase of them in the brain. For instance, IL-1 expression in the brain has been documented after an increase of circulating IL-1 induced by LPS PC (Gabellec et al., 1995). On the other hand, inducers of PC, such as LPS and cytokines, can act through interaction with receptors in circumventricular organs that lack a BBB, triggering upregulation of cytokine levels in the brain. Accordingly, it has been shown that perivascular cells and neurons in the circumventricular organs produce TNF-α in response to systemically administered LPS (Breder et al., 1994).

CHEMOKINES

Chemokines are small molecules involved in the recruitment of immune cells to sites of inflammation or injury (Charo and Ransohoff, 2006). In the central nervous system, chemokines are highly upregulated in neuropathologic conditions. Neurons, astrocytes, microglia, endothelial cells as well as circulating leukocytes are potential sources of chemokines. Several chemokines have been linked to the pathogenesis of ischemic brain injury (Jaerve and Müller, 2012). Upon release, they exacerbate tissue injury by increasing leukocyte and monocytes/macrophages infiltration, potentiating neuronal injury. However, chemokines also possess neuroprotective and neurotrophic functions (Semple et al., 2009). The prevalence of beneficial or detrimental effects may depend on various factors, including the type of the chemokine, its concentration, the time course of production relative to the time of injury and the target cell type (Semple et al., 2009). The contribution of chemokines to PC has been investigated in several models of IT. For instance, HPC or BCCAo prior to transient or global MCAo resulted in reduced infarct size attributed to CCL2 (MCP-1), a leukocyte-derived pro-inflammatory chemokine (Rehni and Singh, 2012; Stowe et al., 2012). Following HPC, expression of CCL2 increased in both neurons and cerebral endothelial cells prior to the induction of the ischemic injury. Moreover, the induction of IT was blocked by CCL2 neutralizing antibodies and was not observed in CCL2-knockout mice (Stowe et al., 2012). Although the protection conferred by HPC is associated with the number of $CCR2^{+}$ monocytes in blood and leaving the circulation, their direct protective role in brain IT needs to be further characterized. Rehni et al. have shown that IT induced by BCCAo PC was lost after treatment with a selective antagonist of CCR2 (Rehni and Singh, 2012), corroborating the role of CCL2 chemokines and its receptor in IT.

CANNABINOIDS

The endocannabinoid system consists of the cannabinoid type 1 (CB1) and type 2 (CB2) receptors and their ligands (Howlett et al., 2002). The expression of these two receptor subtypes varies among tissues. CB1 receptors are abundant in the CNS and are also present in several peripheral tissues, albeit to a lesser extent (Matsuda et al., 1990; Galiègue et al., 1995). Although also present in the brain (Gong et al., 2006), CB2 is mostly expressed in immune cells (Galiègue et al., 1995). Cannabinoid receptors may be activated upon binding of their endogenous ligands arachidonoyl ethanolamide or anandamide (AEA), 2-arachidonoylglycerol (2-AG), and 2-arachidonyl glyceryl ether (noladin ether), or synthetic analogs which are derivatives of herbal cannabinoids, such as the terpenoid Δ^9-tetrahydrocannabinol (THC), the active compound of the cannabis plant (Di Marzo et al., 1998). Both CB1 and CB2 receptors are members of the seven-transmembrane G-protein coupled receptor superfamily. They may initiate downstream signaling pathways that activate potassium channels, phosphatidylinositol-3-kinase and mitogen-activated protein kinases (Di Marzo et al., 1998). The CNS endocannabinoid system has a variety of physiological roles, which are mainly mediated by CB1 receptors. These include, but are not limited to: psychotropic effect, pain inhibition, memory function and increased appetite (Ameri, 1999; Pacher and Haskó, 2008). Modulation of immune responses and the release of inflammatory mediators have been primarily attributed to CB2 receptors (Pacher et al., 2006; Ullrich et al., 2007; Elliott et al., 2011).

The endocannabinoid system has been implicated in ischemic injury (Pacher and Haskó, 2008). Several studies have shown a neuroprotective effect of CB activation in brain ischemia (Nagayama et al., 1999; Panikashvili et al., 2001) by decreasing intracellular Ca^{2+} (Zhuang et al., 2005), modulating brain temperature (Leker et al., 2003), inhibiting pro-inflammatory signaling cascades (Panikashvili et al., 2005) and by preventing endothelial cell activation and leukocyte adhesion (Zhang et al., 2007, 2009). There is emerging evidence that cannabinoids also play a role in PC. Induction of IT by electroacupuncture PC improves neuronal survival in a rat model of focal ischemia (Wang et al., 2005, 2009; Ma et al., 2011). While early PC in this model was dependent on the activation of CB1 receptors as demonstrated by the reversal of the protective effect after administration of the CB1 receptor antagonist AM251 or CB1 siRNA (Wang et al., 2009), delayed PC was dependent upon the activation of CB2 receptors (Ma et al., 2011). It was shown that IT was partially reversed when animals were treated with the specific CB2 antagonist AM630. Inhibition of the CB1 receptor, however, did not block the induction of delayed IT. Although the cellular components of this response have not been investigated, the nearly exclusively expression of CB2 receptors on immune cells makes it possible that modulation of the immune response accounts for the observed neuroprotection.

iNOS

Nitric oxide (NO) is produced by nitric oxidase synthase (NOS) through oxidation of the guanidino nitrogen of L-arginine. Endothelial (eNOS), neuronal (nNOS) and inducible or inflammatory (iNOS) isoforms are found in the brain, and play important roles in IT (for recent review see (Iadecola et al., 2011). iNOS is specifically expressed under pathological conditions, typically those associated with inflammation (Nathan, 1997). Data from our laboratory have shown that iNOS mediates IT following either IPC or LPS-PC in a mouse model of transient MCAo (Cho et al., 2005). PC-mediated neuroprotection was abolished when the selective inhibitor, aminoguanidine, was delivered prior to PC, or when iNOS null mice were used. iNOS expression and accumulation of peroxynitrite in cerebral blood vessels was observed 24 h after IPC (Cho et al., 2005), whereas LPS-PC was associated with accumulation of peroxynitrite in neurons and vessels (Kunz et al., 2007). The role of iNOS in PC has also been reported with anesthetic preconditioning (Kapinya et al., 2002). Prior treatment with isoflurane or halothane, which reduces infarct volume after permanent MCAo, induced iNOS expression in the cortex 18–24 h after PC. Analogous to the studies mentioned above, anesthetic PC was blocked when animals were treated with the iNOS inhibitor aminoguanidine.

COX-2

Similar to iNOS, brain cycloxygenase-2 (COX-2) is markedly upregulated during post-ischemic inflammation and its reaction products contribute to the evolution of ischemic damage (Iadecola et al., 2001). Likewise, the role of COX-2 has been investigated in several PC paradigms. Increased brain levels of COX-2 following PC by CSD, was associated with the development of cerebral IT in a rat model of MCAo (Horiguchi et al., 2006). IPC by transient MCAo induced COX-2 and HO-1 expression, and significantly reduced infarct volume after index MCAo. Administration of the COX-2-selective inhibitor rofecoxib abolished the neuroprotective effect of IPC, indicating a key role of COX-2 in establishing IT (Park et al., 2008). More recently, the role of COX-2 in PC induced by HBO has also been reported (Cheng et al., 2011). COX-2 induction is associated with protection against subsequent global cerebral occlusion in rats and IT was abolished by treatment with COX-2 selective inhibitor NS-398. Contrary to IPC, no role of COX-2 in LPS PC was found in a excitotoxic model of brain injury induced by NMDA (Kawano et al., 2007).

ROS

ROS play a key role in the pathogenesis of the ischemic cascade. ROS are mainly generated after cerebral ischemia by the injured tissue during the acute phase (minutes-hours) and by infiltrating leukocytes during the sub-acute ischemic phase (Chan, 2001; Kahles et al., 2007). For example, neutrophils that infiltrate the ischemic brain produce excessive superoxide via NADPH oxidase, contributing to the exacerbation of ischemic injury (Kunz et al., 2006; Chen et al., 2009; Pun et al., 2009). In preconditioning, the role of ROS via inflammatory mechanisms has also been reported. Kunz et al. showed improvement of cerebrovascular function after transient MCAo in LPS preconditioned mice, an effect associated with peroxynitrite formation (Kunz et al., 2007). Interestingly, peroxynitrite was specifically formed after PC from the reaction of iNOS-derived NO and Nox2 (NADPH oxidase)-derived superoxide, and was not observed in iNOS or Nox2-null

mice. Moreover, peroxynitrite was also found to be beneficial in the tolerance induced by LPS-PC to brain injury resulting from cortical injection with NMDA (Kawano et al., 2007).

EPIGENETIC MECHANISMS

In delayed PC, the time window during which IT is observed can last for days or even weeks. This contrasts with the limited duration of inflammatory signaling where responses are often short lived, and several negative feedback loops are in place to restrict the duration of the inflammatory response. This is achieved by the expression of a series of cellular inhibitors that are concomitantly produced along with pro-inflammatory molecules, leading to a timely termination of gene expression after the stimulus subsides. These inhibitors can directly target transcription factors, as is the case with NF-κB, which is sequestered in the cytoplasm by newly synthetized IκB proteins—or, by inactivating key proteins within the pro-inflammatory signaling cascade. For example, A20, an ubiquitin editing enzyme that is highly inducible by pro-inflammatory stimuli, targets several proteins in the TNFR signaling cascades by removing K63-linked ubiquitin chains from TRAF2, TRAF6 and NEMO. It results in suppression of NF-κB signaling, and by replacing K63- with K48-linked ubiquitin chains in RIP1, thus targeting it for proteasomal degradation (Verstrepen et al., 2010). Similarly, suppressor of cytokine signaling (SOCS), a family of 40 related proteins, and PIAS (protein inhibitor of activated STAT) target primarily the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathway, which is the main cellular transducer of cytokine signals (Yoshimura et al., 2007). Accordingly, short-acting inflammatory signals, as applied during PC protocols, are transient, and not suitable to alter the cellular state for an extended period of time. This view is supported by experimental data in ischemic and LPS preconditioned animals, that show transient global alterations of gene expression profiles after PC, which have largely dissipated by the time of index ischemia (Stenzel-Poore et al., 2003; Marsh et al., 2009b). This raises the question of how the "memory" of the PC event is preserved if the attendant mRNA response has already subsided. An essential feature of such a mechanism, is its persistence beyond the initial stimulation and its decay over time, thus, equipping the cell or organ with a relatively short lived memory of a previous exposure to a potentially lethal stressor. One possibility is that the protein products of the subsided transcriptional activity are still present at the time of induction of ischemia, which could steer the response from injury toward protection. Another possibility is that the transcriptional response to PC induces longer lasting epigenetic changes that shape the genetic response when injurious ischemia occurs. Epigenetic mechanisms include all processes that regulate gene expression without alteration of the underlying DNA sequence, and include, for example, histone modifications, DNA methylation, and non-coding RNAs.

HISTONE AND DNA MODIFICATIONS IN PRECONDITIONING OF THE IMMUNE SYSTEM AND THE BRAIN

Histone modifications by methylation, acetylation, phosphorylation, ubiquitination, and sumoylation are key events in regulating chromatin structure and gene expression. The mechanism by which these modifications alter gene expression is not entirely understood, but may involve tethering of chromatin remodeling machinery, transcriptional repressors and activators. While acetylation of N-terminal histone tails are a hallmark of actively transcribed genes, it is less likely that histone acetylation plays a major role in imprinting a lasting memory from a previous exposure. Supporting this view is the fact that histone deacetylase (HDAC) is recruited early to the promoter of induced genes, in most cases during the gene activation process itself, resulting in a high turnover rate of acetylation marks in the order of minutes and fast disposal of this modification once transcription of the particular gene subsides (Hazzalin and Mahadevan, 2005). On the other hand, DNA methylation is a very thermostable epigenetic mark and, with the possible exception of 5-hydroxymethylcytosine, refractory to environmental stimuli. Therefore, DNA methylation is unsuited to act as an on-off switch for epigenetic memory (Qureshi and Mehler, 2010). Histone H3 tri-methylation at lysine 4 (H3K4me3), in contrast, has been shown to mark active or poised enhancers for an extended period of time. In a model of Candida albicans infected macrophages, persistent H3K4me3 modifications in the enhancer regions of inflammatory genes was associated with a more robust inflammatory response and reduced re-infection rate upon re-exposure (Quintin et al., 2012). Notably, the K4 trimethylation was observed for up to a week after deposition. The importance of H3K4me3 in changing transcriptional responses has been shown in LPS-preconditioned macrophages (Foster et al., 2007). Macrophages pre-exposed to LPS responded to a second LPS exposure with a transcriptional program distinct from that of naïve macrophages exposed to LPS. Genes could be divided into "tolerizeable" genes (T), which were suppressed in tolerant macrophages, and "non-tolerizeable" genes (NT), expressed at higher levels in tolerant than non-tolerant macrophages. It was found that promoter regions of T-class genes were refractory to histone H4 acetylation and H3K4 trimethylation, two modifications positively correlated with transcriptional activity, upon the second LPS challenge (Foster et al., 2007). Depending on the chromatin status in the resting state, genes can be divided in primary and secondary response genes (Ramirez-Carrozzi et al., 2006). Primary genes show a chromatin structure that is suited for immediate transcription and their activation is independent of new protein synthesis. Secondary genes show histone modifications characteristic of inactivated genes and the removal of this block and gene induction is protein synthesis dependent. Interestingly, this study found that LPS-PC changed many genes from secondary to primary response genes upon LPS re-stimulation, further implicating that epigenetic memory has been established in this model.

Several histone modifiers have been implicated in the PC response of the brain, including sirtuin family HDACs (Morris et al., 2011) and polycomb group proteins (PcG), a multimeric protein complex that mediates gene silencing and PC (Stapels et al., 2010). In addition to their role in neuronal physiology, both protein families have been implicated in the regulation of immune function (Swigut and Wysocka, 2007; Gallí et al., 2011). Whether they also regulate the immune signaling during PC, however, has not been directly addressed thus far.

MICRORNAS AND LONG NON-CODING RNAS

microRNAs (miRNAs) are small non-coding RNAs (18- to 24- nucleotides) that repress translation of mRNAs. Mature miRNAs are excised from a precursor RNA by the activity of Dicer and are guided to the 3′-untranslated region (3′-UTR) of mRNAs targets by the RNA-induced silencing complex (RISC). This leads to the down-regulation of gene expression via degradation or translational inhibition (Bartel, 2009). miRNAs have an essential quality that makes them compatible with the maintenance of preconditioning memory, namely their stability (Rüegger and Großhans, 2012). In addition, they are inducible by environmental stimuli, and are regulated at the levels of transcription, biogenesis, stability and decay. Supportive for a role of some miRNAs in establishing epigenetic memory, is the fact that they are extremely long lived, more than 12 days in the case of miR-208 in the heart (van Rooij et al., 2007). Furthermore, ablation of many miRNAs is well tolerated in the mouse, with these mutant animals only developing substantially altered phenotypes after being subjected to some kind of stress. These observations suggest that the persistence of inducible miRNAs due to their long half-lives after initial stimulation, may enable the maintenance of gene-expression programs that enhance the resistance of cells to repeated stress-exposure. When such "memory" miRNAs are absent (such as in animals depleted of the genes encoding the miRNAs), aberrant phenotypes become apparent (Leung and Sharp, 2010). The findings that miRNA can be actively secreted and transferred from cell-to-cell via microvesicles makes it possible that miRNA can work at a distance by transferring epigenetic signatures from a stimulus-exposed cell to a cell that has not encountered the particular environmental stressor (Valadi et al., 2007).

It is estimated that 500–1000 different miRNAs are expressed in mammalian cells and several miRNAs have been implicated in the regulation of the immune response and inflammation (O'Connell et al., 2012). Certain miRNAs, for instance, miR-9, -21, -146a, -147, -203, are induced in response to TLR activation, and serve as negative feedback regulators of the MyD88-dependent TLR signaling pathway and NF-κB activity (O'Neill et al., 2011). Conversely, miR-155 enhances the inflammatory response by down-regulating negative regulators Src Homology-2 domain-containing inositol-5′-phosphatase 1 (SHIP1) and SOCS1 leading to prolonged activation of AKT and IFN signaling pathways (O'Connell et al., 2009).

Cerebral ischemia-reperfusion injury alters the expression of several miRNAs (Dharap et al., 2009; Wang et al., 2013). Interestingly, IPC and HPC have been shown to rapidly alter miRNA levels in the brain (Dharap and Vemuganti, 2010; Lee et al., 2010; Liu et al., 2012). Although it is unknown if these alterations directly affect immune pathways participating in PC, the finding that expression of miRNAs involved in inflammatory signaling is altered by IPC in mice, is suggestive for such a role. For example, miR-17-5p, an inhibitor of monocyte maturation, and the TLR4-targeting miRNA let-7e were down-regulated in the cerebral cortex of mice after IPC (Lusardi et al., 2010). Furthermore, the peroxisome proliferator-activated receptor gamma (PPARγ)-targeting miR-27b and miR-146a, which regulates Traf6, IRAK1, and IRAK2 expression, were up-regulated after PC by transient MCAo in rats (Dharap and Vemuganti, 2010). The same study showed down-regulation of miR-145, which targets the essential TLR4-associated signaling molecule MyD88-adapter-like (MAL). Tellingly, monocyte chemotactic protein-induced protein 1 (MCPIP1), a multifunctional protein with RNAse activity that antagonizes Dicer, and thereby inhibits miRNA biogenesis (Suzuki et al., 2011), has been shown to abrogate the effects of LPS-PC in a mouse model of transient cerebral ischemia (Liang et al., 2011). Taken together, although PC-induced regulation of immune components by miRNA has not been conclusively shown to contribute to cerebral IT, the correlative data gathered so far are supportive for a role of miRNAs in regulation of immune pathways during PC.

Long non-coding RNAs range from 200 bp to several kb, and are expressed in a cell type specific manner (Mercer et al., 2009). Although most of these molecules have not been functionally characterized, they may have diverse roles ranging from regulation of chromosome inactivation to control of gene expression (Mercer et al., 2009). Because several long non-coding RNAs have been shown to be inducible and are involved in regulating immune cell activity and inflammatory signaling, they constitute another potential player by which epigenetic processes can establish a cellular memory. Their role in PC has yet to be investigated.

CONCLUSIONS

Although post-ischemic inflammation is considered a major pathogenic factor in stroke, emerging evidence supports a beneficial role of selected inflammatory pathways. One such mechanism is the induction of endogenous neuroprotection through ischemic PC (**Figure 3**). The evidence summarized in this review indicates that the immune system plays a central role in the mechanisms eliciting neuroprotective responses after PC. While activation of IT-inducing immune pathways shows robust neuroprotection in animal models, the clinical translation of such approaches is complicated by side effects including systemic inflammation and immunodepression (Meisel et al., 2005; McColl et al., 2009). The identification of specific pathways and effector molecules involved in establishing inflammatory PC will help to design targeted PC protocols that can be potentially translated into clinical practice (Keep et al., 2010; Anrather and Hallenbeck, 2013; Narayanan et al., 2013). Notably, immune pathways are not only involved in PC induced by inflammatory mediators, but have also been implicated in several PC paradigms that are not triggered by inflammatory signals, such as ischemic and anesthetic PC, attesting a pivotal role for the immune system in mediating and/or establishing IT. Because of the involvement of inflammatory pathways in a wide variety of diseases, many of the immune components involved in PC are prime therapeutic targets and subjects of extensive drug development. Several proteins involved in PC, such as type I IFNs, are currently in clinical use, and re-purposing them for stroke therapy or prevention could provide a fast track for clinical translation. However, much remains to be learned. For example, few studies have investigated the role of specific immune cells in IT, an area of research that could result in cell-specific therapies and would therefore mitigate the likelihood of adverse effects associated with administration

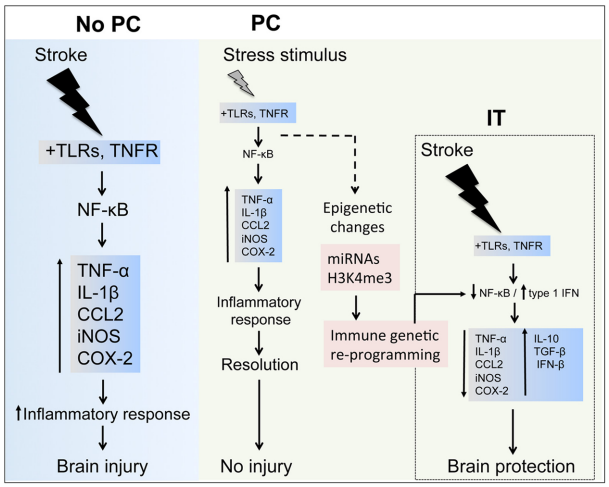


FIGURE 3 | Inflammatory components in stroke and preconditioning. Stroke induces a major inflammatory response through toll-like receptors (TLRs) and tumor necrosis factor receptor (TNFR) signaling, which activate nuclear factor-κB (NF-κB) resulting in upregulation of inflammatory molecules, such as TNF-α, interleukin (IL)-1β, CCL2, inducible nitric oxidase synthase (iNOS) and cyclooxygenase (COX)-2, contributing to the ischemic brain injury. In preconditioning (PC), the exposure to a wide range of stressors activates inflammatory pathways and leads to upregulation of inflammatory molecules similarly to those induced by stroke. After the effects of the stressor have subsided, the inflammatory response is resolved during the early phase of PC and gene expression returns to basal levels. However, longer lasting epigenetic changes induced in the immune system components by micro-RNAs (miRNAs) and histone methylation (i.e., histone H3 trimethylation at lysine 4, H3K4me3) may reprogram inflammatory pathways to respond differently after an episode of severe ischemia, for instance favoring the expression of anti-inflammatory cytokines (IL-10, tumor growth factor-(TGF)-β and interferon-(IFN)-β that induces ischemic tolerance (IT).

of inflammatory cytokines. In addition, a better understanding of the epigenetic mechanisms involved in IT could lead to therapies that preserve PC-induced epigenetic changes, thereby substantially extending the time frame in which IT is observed.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) Grants NS34179 (Costantino Iadecola) and NS081179 (Josef Anrather). Corinne Benakis was supported by grant P3SMP3 148367 from the Swiss National Science Foundation and the Swiss Foundation for Grants in Biology and Medicine (SFGBM).

REFERENCES

Abe, T., Shimamura, M., Jackman, K., Kurinami, H., Anrather, J., Zhou, P., et al. (2010). Key role of CD36 in Toll-like receptor 2 signaling in cerebral ischemia. *Stroke* 41, 898–904. doi: 10.1161/STROKEAHA.109.572552

Ahmed, S. H., He, Y. Y., Nassief, A., Xu, J., Xu, X. M., Hsu, C. Y., et al. (2000). Effects of lipopolysaccharide priming on acute ischemic brain injury. *Stroke* 31, 193–199. doi: 10.1161/01.STR.31.1.193

Akira, S. (2009). Pathogen recognition by innate immunity and its signaling. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 85, 143–156. doi: 10.2183/pjab.85.143

Allan, S. M., Tyrrell, P. J., and Rothwell, N. J. (2005). Interleukin-1 and neuronal injury. *Nat. Rev. Immunol.* 5, 629–640. doi: 10.1038/nri1664

Ameri, A. (1999). The effects of cannabinoids on the brain. *Prog. Neurobiol.* 58, 315–348. doi: 10.1016/S0301-0082(98)00087-2

Anderson, K. V., Bokla, L., and Nüsslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell* 42, 791–798. doi: 10.1016/0092-8674(85)90275-2

Aneja, R., Odoms, K., Dunsmore, K., Shanley, T. P., and Wong, H. R. (2006). Extracellular heat shock protein-70 induces endotoxin tolerance in THP-1 cells. *J. Immunol.* 177, 7184–7192.

Anrather, J., and Hallenbeck, J. M. (2013). Biological networks in ischemic tolerance—rethinking the approach to clinical conditioning. *Transl. Stroke Res.* 4, 114–129. doi: 10.1007/s12975-012-0244-z

Baeuerle, P. A., and Henkel, T. (1994). Function and activation of NF-kappaB in the immune system. *Annu. Rev. Immunol.* 12, 141–179. doi: 10.1146/annurev.iy.12.040194.001041

Baldwin, A. S. (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14, 649–683. doi: 10.1146/annurev.immunol.14.1.649

Banks, W. A. (2005). Blood-brain barrier transport of cytokines: a mechanism for neuropathology. *Curr. Pharm. Des.* 11, 973–984. doi: 10.2174/1381612053381684

Barone, F. C., White, R. F., Spera, P. A., Ellison, J., Currie, R. W., Wang, X., et al. (1998). Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* 29, 1937–1950. discussion: 1950–1951.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002

Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukocyte Biol.* 81, 1–5. doi: 10.1189/jlb.0306164

Bigdeli, M. R., and Khoshbaten, A. (2008). *In vivo* preconditioning with normobaric hyperoxia induces ischemic tolerance partly by triggering tumor necrosis factor-alpha converting enzyme/tumor necrosis factor-alpha/nuclear factor-kappaB. *Neuroscience* 153, 671–678. doi: 10.1016/j.neuroscience.2008.02.064

Blondeau, N., Widmann, C., Lazdunski, M., and Heurteaux, C. (2001). Activation of the nuclear factor-kappaB is a key event in brain tolerance. *J. Neurosci.* 21, 4668–4677.

Brea, D., Blanco, M., Ramos-Cabrer, P., Moldes, O., Arias, S., Pérez-Mato, M., et al. (2011). Toll-like receptors 2 and 4 in ischemic stroke: outcome and therapeutic values. *J. Cereb. Blood Flow Metab.* 31, 1424–1431. doi: 10.1038/jcbfm.2010.231

Breder, C. D., Hazuka, C., Ghayur, T., Klug, C., Huginin, M., Yasuda, K., et al. (1994). Regional induction of tumor necrosis factor alpha expression in the mouse brain after systemic lipopolysaccharide administration. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11393–11397. doi: 10.1073/pnas.91.24.11393

Cao, C.-X., Yang, Q.-W., Lv, F.-L., Cui, J., Fu, H.-B., and Wang, J.-Z. (2007). Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. *Biochem. Biophys. Res. Commun.* 353, 509–514. doi: 10.1016/j.bbrc.2006.12.057

Cárdenas, A., Moro, M. A., Leza, J. C., O'Shea, E., Dávalos, A., Castillo, J., et al. (2002). Upregulation of TACE/ADAM17 after ischemic preconditioning is involved in brain tolerance. *J. Cereb. Blood Flow Metab.* 22, 1297–1302. doi: 10.1097/00004647-200211000-00002

Ceulemans, A.-G. A., Zgavc, T. T., Kooijman, R. R., Hachimi-Idrissi, S. S., Sarre, S. S., and Michotte, Y. Y. (2010). The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. *J. Neuroinflammation* 7, 74–74. doi: 10.1186/1742-2094-7-74

Chan, P. H. (2001). Reactive oxygen radicals in signaling and damage in the ischemic brain. *J. Cereb. Blood Flow Metab.* 21, 2–14. doi: 10.1097/00004647-200101000-00002

Charo, I. F., and Ransohoff, R. M. (2006). The many roles of chemokines and chemokine receptors in inflammation. *N. Engl. J. Med.* 354, 610–621. doi: 10.1056/NEJMra052723

Chen, G. Y., and Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* 10, 826–837. doi: 10.1038/nri2873

Chen, H., Song, Y. S., and Chan, P. H. (2009). Inhibition of NADPH oxidase is neuroprotective after ischemia-reperfusion. *J. Cereb. Blood Flow Metab.* 29, 1262–1272. doi: 10.1038/jcbfm.2009.47

Cheng, O., Ostrowski, R. P., Wu, B., Liu, W., Chen, C., and Zhang, J. H. (2011). Cyclooxygenase-2 mediates hyperbaric oxygen preconditioning in the rat model of transient global cerebral ischemia. *Stroke* 42, 484–490. doi: 10.1161/STROKEAHA.110.604421

Cho, S., Park, E.-M., Zhou, P., Frys, K., Ross, M. E., and Iadecola, C. (2005). Obligatory role of inducible nitric oxide synthase in ischemic preconditioning. *J. Cereb. Blood Flow Metab.* 25, 493–501. doi: 10.1038/sj.jcbfm.9600058

Clarkson, A. N. (2007). Anesthetic-mediated protection/preconditioning during cerebral ischemia. *Life Sci.* 80, 1157–1175. doi: 10.1016/j.lfs.2006.12.022

Clemens, J. A., Stephenson, D. T., Yin, T., Smalstig, E. B., Panetta, J. A., Little, S. P., et al. (1998). Drug-induced neuroprotection from global ischemia is associated with prevention of persistent but not transient activation of nuclear factor-B in rats editorial comment. *Stroke* 29, 677–682. doi: 10.1161/01.STR.29.3.677

Curry, A., Guo, M., Patel, R., Liebelt, B., Sprague, S., Lai, Q., et al. (2010). Exercise pre-conditioning reduces brain inflammation in stroke via tumor necrosis factor-α, extracellular signal-regulated kinase 1/2 and matrix metalloproteinase-9 activity. *Neurol. Res.* 32, 756–762. doi: 10.1179/174313209 X459101

Dawn, B., Xuan, Y.-T., Guo, Y., Rezazadeh, A., Stein, A. B., Hunt, G., et al. (2004). IL-6 plays an obligatory role in late preconditioning via JAK-STAT signaling and upregulation of iNOS and COX-2. *Cardiovasc. Res.* 64, 61–71. doi: 10.1016/j.cardiores.2004.05.011

Dharap, A., Bowen, K., Place, R., Li, L.-C., and Vemuganti, R. (2009). Transient focal ischemia induces extensive temporal changes in rat cerebral MicroRNAome. *J. Cereb. Blood Flow Metab.* 29, 675–687. doi: 10.1038/jcbfm.2008.157

Dharap, A., and Vemuganti, R. (2010). Ischemic pre-conditioning alters cerebral microRNAs that are upstream to neuroprotective signaling pathways. *J. Neurochem.* 113, 1685–1691. doi: 10.1111/j.1471-4159.2010.06735.x

Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, L. (1998). Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci.* 21, 521–528. doi: 10.1016/S0166-2236(98)01283-1

Ding, Y.-H., Young, C. N., Luan, X., Li, J., Rafols, J. A., Clark, J. C., et al. (2005). Exercise preconditioning ameliorates inflammatory injury in ischemic rats during reperfusion. *Acta Neuropathol.* 109, 237–246. doi: 10.1007/s00401-004-0943-y

Dirnagl, U., and Meisel, A. (2008). Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? *Neuropharmacology* 55, 334–344. doi: 10.1016/j.neuropharm.2008.02.017

Downes, C. E., and Crack, P. J. (2010). Neural injury following stroke: are Toll-like receptors the link between the immune system and the CNS? *Br. J. Pharmacol.* 160, 1872–1888. doi: 10.1111/j.1476-5381.2010.00864.x

Du, F., Zhu, L., Qian, Z.-M., Wu, X.-M., Yung, W.-H., and Ke, Y. (2010). Hyperthermic preconditioning protects astrocytes from ischemia/reperfusion injury by up-regulation of HIF-1 alpha expression and binding activity. *Biochim. Biophys. Acta* 1802, 1048–1053. doi: 10.1016/j.bbadis.2010.06.013

Duckworth, E. A. M., Butler, T., Collier, L., Collier, S., and Pennypacker, K. R. (2006). NF-κB protects neurons from ischemic injury after middle cerebral artery occlusion in mice. *Brain Res.* 1088, 167–175. doi: 10.1016/j.brainres.2006.02.103

Elliott, M. B., Tuma, R. F., Amenta, P. S., Barbe, M. F., and Jallo, J. I. (2011). Acute effects of a selective cannabinoid-2 receptor agonist on neuroinflammation in a model of traumatic brain injury. *J. Neurotrauma* 28, 973–981. doi: 10.1089/neu.2010.1672

Endres, M., Gertz, K., Lindauer, U., Katchanov, J., Schultze, J., Schröck, H., et al. (2003). Mechanisms of stroke protection by physical activity. *Ann. Neurol.* 54, 582–590. doi: 10.1002/ana.10722

Foster, S. L., Hargreaves, D. C., and Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 447, 972–978. doi: 10.1038/nature05836

Gabellec, M. M., Griffais, R., Fillion, G., and Haour, F. (1995). Expression of interleukin 1 alpha, interleukin 1 beta and interleukin 1 receptor antagonist mRNA in mouse brain: regulation by bacterial lipopolysaccharide (LPS) treatment. *Brain Res. Mol. Brain Res.* 31, 122–130. doi: 10.1016/0169-328X(95)00042-Q

Galea, E., Glickstein, S. B., Feinstein, D. L., Golanov, E. V., and Reis, D. J. (1998a). Stimulation of cerebellar fastigial nucleus inhibits interleukin-1beta-induced cerebrovascular inflammation. *Am. J. Physiol.* 275, H2053–H2063.

Galea, E., Golanov, E. V., Feinstein, D. L., Kobylarz, K. A., Glickstein, S. B., and Reis, D. J. (1998b). Cerebellar stimulation reduces inducible nitric oxide synthase expression and protects brain from ischemia. *Am. J. Physiol.* 274, H2035–H2045.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* 232, 54–61. doi: 10.1111/j.1432-1033.1995.tb20780.x

Gallí, M., Van Gool, F., and Leo, O. (2011). Sirtuins and inflammation: friends or foes? *Biochem. Pharmacol.* 81, 569–576. doi: 10.1016/j.bcp.2010.12.010

Gesuete, R., Packard, A. E. B., Vartanian, K. B., Conrad, V. K., Stevens, S. L., Bahjat, F. R., et al. (2012). Poly-ICLC preconditioning protects the blood-brain barrier against ischemic injury *in vitro* through type I interferon signaling. *J. Neurochem.* 123(Suppl. 2), 75–85. doi: 10.1111/j.1471-4159.2012.07946.x

Gidday, J. M. (2006). Cerebral preconditioning and ischaemic tolerance. *Nat. Rev. Neurosci.* 7, 437–448. doi: 10.1038/nrn1927

Ginis, I., Jaiswal, R., Klimanis, D., Liu, J., Greenspon, J., and Hallenbeck, J. M. (2002). TNF-alpha-induced tolerance to ischemic injury involves differential control of NF-kappaB transactivation: the role of NF-kappaB association with p300 adaptor. *J. Cereb. Blood Flow Metab.* 22, 142–152. doi: 10.1097/00004647-200202000-00002

Golanov, E. V., Liu, F., and Reis, D. J. (1998). Stimulation of cerebellum protects hippocampal neurons from global ischemia. *Neuroreport* 9, 819–824. doi: 10.1097/00001756-199803300-00010

Gong, J.-P., Onaivi, E. S., Ishiguro, H., Liu, Q.-R., Tagliaferro, P. A., Brusco, A., et al. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res.* 1071, 10–23. doi: 10.1016/j.brainres.2005.11.035

Guo, M., Lin, V., Davis, W., Huang, T., Carranza, A., Sprague, S., et al. (2008). Preischemic induction of TNF-alpha by physical exercise reduces blood-brain barrier dysfunction in stroke. *J. Cereb. Blood Flow Metab.* 28, 1422–1430. doi: 10.1038/jcbfm.2008.29

Hallenbeck, J. M. (2002). The many faces of tumor necrosis factor in stroke. *Nat. Med.* 8, 1363–1368. doi: 10.1038/nm1202-1363

Harari, O. A., and Liao, J. K. (2010). NF-κB and innate immunity in ischemic stroke. *Ann. N.Y. Acad. Sci.* 1207, 32–40. doi: 10.1111/j.1749-6632.2010.05735.x

Hazzalin, C. A., and Mahadevan, L. C. (2005). Dynamic acetylation of all lysine 4-methylated histone H3 in the mouse nucleus: analysis at c-fos and c-jun. *PLoS Biol.* 3:e393. doi: 10.1371/journal.pbio.0030393

Hess, D. C., Hoda, M. N., and Bhatia, K. (2013). Remote limb perconditioning and postconditioning: will it translate into a promising treatment for acute stroke? *Stroke* 44, 1191–1196. doi: 10.1161/STROKEAHA.112.678482

Hickey, E. J., You, X., Kaimaktchiev, V., Stenzel-Poore, M., and Ungerleider, R. M. (2007). Lipopolysaccharide preconditioning induces robust protection against brain injury resulting from deep hypothermic circulatory arrest. *J. Thorac. Cardiovasc. Surg.* 133, 1588–1596. doi: 10.1016/j.jtcvs.2006.12.056

Horiguchi, T., Snipes, J. A., Kis, B., Shimizu, K., and Busija, D. W. (2005). The role of nitric oxide in the development of cortical spreading depression-induced tolerance to transient focal cerebral ischemia in rats. *Brain Res.* 1039, 84–89. doi: 10.1016/j.brainres.2005.01.047

Horiguchi, T., Snipes, J. A., Kis, B., Shimizu, K., and Busija, D. W. (2006). Cyclooxygenase-2 mediates the development of cortical spreading depression-induced tolerance to transient focal cerebral ischemia in rats. *Neuroscience* 140, 723–730. doi: 10.1016/j.neuroscience.2006.02.025

Hoshino, K., Sugiyama, T., Matsumoto, M., Tanaka, T., Saito, M., Hemmi, H., et al. (2006). IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. *Nature* 440, 949–953. doi: 10.1038/nature04641

Howlett, A. C. A., Barth, F. F., Bonner, T. I. T., Cabral, G. G., Casellas, P. P., Devane, W. A. W., et al. (2002). International union of pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* 54, 161–202. doi: 10.1124/pr.54.2.161

Hu, X., Jiang, H., Cui, B., Xu, C., Lu, Z., and He, B. (2010). Preconditioning with high mobility group box 1 protein protects against myocardial ischemia-reperfusion injury. *Int. J. Cardiol.* 145, 111–112. doi: 10.1016/j.ijcard.2009.05.057

Hua, F., Ma, J., Ha, T., Kelley, J., Williams, D. L., Kao, R. L., et al. (2008). Preconditioning with a TLR2 specific ligand increases resistance to cerebral ischemia/reperfusion injury. *J. Neuroimmunol.* 199, 75–82. doi: 10.1016/j.jneuroim.2008.05.009

Huang, J., Upadhyay, U. M., and Tamargo, R. J. (2006). Inflammation in stroke and focal cerebral ischemia. *Surg. Neurol.* 66, 232–245. doi: 10.1016/j.surneu.2005.12.028

Iadecola, C., and Anrather, J. (2011a). The immunology of stroke: from mechanisms to translation. *Nat. Med.* 17, 796–808. doi: 10.1038/nm.2399

Iadecola, C., and Anrather, J. (2011b). Stroke research at a crossroad: asking the brain for directions. *Nat. Neurosci.* 14, 1363–1368. doi: 10.1038/nn.2953

Iadecola, C., Kahles, T., Gallo, E. F., and Anrather, J. (2011). Neurovascular protection by ischaemic tolerance: role of nitric oxide. *J. Physiol. (Lond.)* 589, 4137–4145. doi: 10.1113/jphysiol.2011.210831

Iadecola, C., Niwa, K., Nogawa, S., Zhao, X., Nagayama, M., Araki, E., et al. (2001). Reduced susceptibility to ischemic brain injury and N-methyl-D-aspartate-mediated neurotoxicity in cyclooxygenase-2-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1294–1299. doi: 10.1073/pnas.98.3.1294

Ikeda, T., Xia, X. Y., Xia, Y. X., and Ikenoue, T. (1999). Hyperthermic preconditioning prevents blood-brain barrier disruption produced by hypoxia-ischemia in newborn rat. *Brain Res. Dev. Brain Res.* 117, 53–58. doi: 10.1016/S0165-3806(99)00097-8

Izuishi, K., Tsung, A., Jeyabalan, G., Critchlow, N. D., Li, J., Tracey, K. J., et al. (2006). Cutting edge: high-mobility group box 1 preconditioning protects against liver ischemia-reperfusion injury. *J. Immunol.* 176, 7154–7158.

Jaerve, A., and Müller, H. W. (2012). Chemokines in CNS injury and repair. *Cell Tissue Res.* 349, 229–248. doi: 10.1007/s00441-012-1427-3

Jander, S., Schroeter, M., Peters, O., Witte, O. W., and Stoll, G. (2001). Cortical spreading depression induces proinflammatory cytokine gene expression in the rat brain. *J. Cereb. Blood Flow Metab.* 21, 218–225. doi: 10.1097/00004647-200103000-00005

Jung, F., Palmer, L. A., Zhou, N., and Johns, R. A. (2000). Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. *Circ. Res.* 86, 319–325. doi: 10.1161/01.RES.86.3.319

Kahles, T., Luedike, P., Endres, M., Galla, H.-J., Steinmetz, H., Busse, R., et al. (2007). NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke* 38, 3000–3006. doi: 10.1161/STROKEAHA.107.489765

Kaltschmidt, B., Widera, D., and Kaltschmidt, C. (2005). Signaling via NF-kappaB in the nervous system. *Biochim. Biophys. Acta* 1745, 287–299. doi: 10.1016/j.bbamcr.2005.05.009

Kaltschmidt, C., Kaltschmidt, B., Neumann, H., Wekerle, H., and Baeuerle, P. A. (1994). Constitutive NF-kappa B activity in neurons. *Mol. Cell Biol.* 14, 3981–3992.

Kapinya, K. J., Löwl, D., Fütterer, C., Maurer, M., Waschke, K. F., Isaev, N. K., et al. (2002). Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent. *Stroke* 33, 1889–1898. doi: 10.1161/01.STR.0000020092.41820.58

Kawahara, N., Ruetzler, C. A., and Klatzo, I. (1995). Protective effect of spreading depression against neuronal damage following cardiac arrest cerebral ischaemia. *Neurol. Res.* 17, 9–16.

Kawahara, N., Ruetzler, C. A., Mies, G., and Klatzo, I. (1999). Cortical spreading depression increases protein synthesis and upregulates basic fibroblast growth factor. *Exp. Neurol.* 158, 27–36. doi: 10.1006/exnr.1999.7091

Kawai, N. N., Okauchi, M. M., Morisaki, K. K., and Nagao, S. S. (2000). Effects of delayed intraischemic and postischemic hypothermia on a focal model of transient cerebral ischemia in rats. *Stroke* 31, 1982–1989. doi: 10.1161/01.STR.31.8.1982

Kawano, T., Kunz, A., Abe, T., Girouard, H., Anrather, J., Zhou, P., et al. (2007). iNOS-derived NO and nox2-derived superoxide confer tolerance to excitotoxic brain injury through peroxynitrite. *J. Cereb. Blood Flow Metab.* 27, 1453–1462. doi: 10.1038/sj.jcbfm.9600449

Keep, R. F., Wang, M. M., Xiang, J., Hua, Y., and Xi, G. (2010). Is there a place for cerebral preconditioning in the clinic? *Transl. Stroke Res.* 1, 4–18. doi: 10.1007/s12975-009-0007-7

Kim, J. B. (2006). HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *J. Neurosci.* 26, 6413–6421. doi: 10.1523/JNEUROSCI.3815-05.2006

Kirino, T. (2002). Ischemic tolerance. *J. Cereb. Blood Flow Metab.* 22, 1283–1296. doi: 10.1097/00004647-200211000-00001

Kitagawa, K., Matsumoto, M., Tagaya, M., Hata, R., Ueda, H., Niinobe, M., et al. (1990). "Ischemic tolerance" phenomenon found in the brain. *Brain Res.* 528, 21–24. doi: 10.1016/0006-8993(90)90189-I

Konstantinov, I. E., Arab, S., Kharbanda, R. K., Li, J., Cheung, M. M. H., Cherepanov, V., et al. (2004). The remote ischemic preconditioning stimulus modifies inflammatory gene expression in humans. *Physiol. Genomics* 19, 143–150. doi: 10.1152/physiolgenomics.00046.2004

Kunkler, P. E., Hulse, R. E., and Kraig, R. P. (2004). Multiplexed cytokine protein expression profiles from spreading depression in hippocampal organotypic cultures. *J. Cereb. Blood Flow Metab.* 24, 829–839. doi: 10.1097/01.WCB.0000126566.34753.30

Kunz, A., Anrather, J., Zhou, P., Orio, M., and Iadecola, C. (2006). Cyclooxygenase-2 does not contribute to postischemic production of reactive oxygen species. *J. Cereb. Blood Flow Metab.* 27, 545–551. doi: 10.1038/sj.jcbfm.9600369

Kunz, A., Park, L., Abe, T., Gallo, E. F., Anrather, J., Zhou, P., et al. (2007). Neurovascular protection by ischemic tolerance: role of nitric oxide and reactive oxygen species. *J. Neurosci.* 27, 7083–7093. doi: 10.1523/JNEUROSCI.1645-07.2007

Lee, S.-T., Chu, K., Jung, K.-H., Yoon, H.-J., Jeon, D., Kang, K.-M., et al. (2010). MicroRNAs induced during ischemic preconditioning. *Stroke* 41, 1646–1651. doi: 10.1161/STROKEAHA.110.579649

Leker, R. R., Gai, N., Mechoulam, R., and Ovadia, H. (2003). Drug-induced hypothermia reduces ischemic damage: effects of the cannabinoid HU-210. *Stroke* 34, 2000–2006. doi: 10.1161/01.STR.0000079817.68944.1E

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86, 973–983. doi: 10.1016/S0092-8674(00)80172-5

Leung, A. K. L., and Sharp, P. A. (2010). MicroRNA functions in stress responses. *Mol. Cell* 40, 205–215. doi: 10.1016/j.molcel.2010.09.027

Leung, P. Y., Packard, A. E., and Stenzel-Poore, M. P. (2009). It's all in the family: multiple Toll-like receptors offer promise as novel therapeutic targets for stroke neuroprotection. *Future Neurol.* 4, 201–208. doi: 10.2217/14796708.4.2.201

Leung, P. Y., Stevens, S. L., Packard, A. E. B., Lessov, N. S., Yang, T., Conrad, V. K., et al. (2012). Toll-like receptor 7 preconditioning induces robust neuroprotection against stroke by a novel type I interferon-mediated mechanism. *Stroke* 43, 1383–1389. doi: 10.1161/STROKEAHA.111.641522

Li, Q., and Verma, I. M. (2002). NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* 2, 725–734. doi: 10.1038/nri910

Li, Q.-F., Zhu, Y.-S., Jiang, H., Xu, H., and Sun, Y. (2009). Heme oxygenase-1 mediates the anti-inflammatory effect of isoflurane preconditioning in LPS-stimulated macrophages. *Acta Pharmacol. Sin.* 30, 228–234. doi: 10.1038/aps.2008.19

Liang, J., Wang, J., Saad, Y., Warble, L., Becerra, E., and Kolattukudy, P. E. (2011). Participation of MCP-induced protein 1 in lipopolysaccharide preconditioning-induced ischemic stroke tolerance by regulating the expression of proinflammatory cytokines. *J. Neuroinflammation* 8:182. doi: 10.1186/1742-2094-8-182

Liu, C., Peng, Z., Zhang, N., Yu, L., Han, S., Li, D., et al. (2012). Identification of differentially expressed microRNAs and their PKC-isoform specific gene network prediction during hypoxic pre-conditioning and focal cerebral ischemia of mice. *J. Neurochem.* 120, 830–841. doi: 10.1111/j.1471-4159.2011.07624.x

Liu, J., Ginis, I., Spatz, M., and Hallenbeck, J. M. (2000). Hypoxic preconditioning protects cultured neurons against hypoxic stress via TNF-alpha and ceramide. *Am. J. Physiol. Cell Physiol.* 278, C144–C153.

Loddick, S. A., Turnbull, A. V., and Rothwell, N. J. (1998). Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. *J. Cereb. Blood Flow Metab.* 18, 176–179. doi: 10.1097/00004647-199802000-00008

Lu, C., Liu, L., Chen, Y., Ha, T., Kelley, J., Schweitzer, J., et al. (2011). TLR2 ligand induces protection against cerebral ischemia/reperfusion injury via activation of phosphoinositide 3-kinase/Akt signaling. *J. Immunol.* 187, 1458–1466. doi: 10.4049/jimmunol.1003428

Lusardi, T. A., Farr, C. D., Faulkner, C. L., Pignataro, G., Yang, T., Lan, J., et al. (2010). Ischemic preconditioning regulates expression of microRNAs and a predicted target, MeCP2, in mouse cortex. *J. Cereb. Blood Flow Metab.* 30, 744–756. doi: 10.1038/jcbfm.2009.253

Ma, L., Zhu, Z., Zhao, Y., Hou, L., Wang, Q., Xiong, L., et al. (2011). Cannabinoid receptor type 2 activation yields delayed tolerance to focal cerebral ischemia. *Curr. Neurovasc. Res.* 8, 145–152. doi: 10.2174/156720211795495394

Macrez, R., Ali, C., Toutirais, O., Le Mauff, B., Defer, G., Dirnagl, U., et al. (2011). Stroke and the immune system: from pathophysiology to new therapeutic strategies. *Lancet Neurol.* 10, 471–480. doi: 10.1016/S1474-4422(11)70066-7

Mallard, C. (2012). Innate immune regulation by toll-like receptors in the brain. *ISRN Neurol.* 2012:701950. doi: 10.5402/2012/701950

Marsh, B., Stevens, S. L., Packard, A. E. B., Gopalan, B., Hunter, B., Leung, P. Y., et al. (2009b). Systemic lipopolysaccharide protects the brain from ischemic injury

by reprogramming the response of the brain to stroke: a critical role for IRF3. *J. Neurosci.* 29, 9839–9849. doi: 10.1523/JNEUROSCI.2496-09.2009

Marsh, B. J., Williams-Karnesky, R. L., and Stenzel-Poore, M. P. (2009a). Toll-like receptor signaling in endogenous neuroprotection and stroke. *Neuroscience* 158, 1007–1020. doi: 10.1016/j.neuroscience.2008.07.067

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346, 561–564. doi: 10.1038/346561a0

McColl, B. W., Allan, S. M., and Rothwell, N. J. (2009). Systemic infection, inflammation and acute ischemic stroke. *Neuroscience* 158, 1049–1061. doi: 10.1016/j.neuroscience.2008.08.019

Meisel, C., Schwab, J. M., Prass, K., Meisel, A., and Dirnagl, U. (2005). Central nervous system injury-induced immune deficiency syndrome. *Nat. Rev. Neurosci.* 6, 775–786. doi: 10.1038/nrn1765

Mercer, T. R., Dinger, M. E., and Mattick, J. S. (2009). Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* 10, 155–159. doi: 10.1038/nrg2521

Morris, K. C., Lin, H. W., Thompson, J. W., and Perez-Pinzon, M. A. (2011). Pathways for ischemic cytoprotection: role of sirtuins in caloric restriction, resveratrol, and ischemic preconditioning. *J. Cereb. Blood Flow Metab.* 31, 1003–1019. doi: 10.1038/jcbfm.2010.229

Nagayama, T. T., Sinor, A. D. A., Simon, R. P. R., Chen, J. J., Graham, S. H. S., Jin, K. K., et al. (1999). Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J. Neurosci.* 19, 2987–2995.

Narayanan, S. V., Dave, K. R., and Perez-Pinzon, M. A. (2013). Ischemic preconditioning and clinical scenarios. *Curr. Opin. Neurol.* 26, 1–7. doi: 10.1097/WCO.0b013e32835bf200

Nathan, C. (1997). Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest.* 100, 2417–2423. doi: 10.1172/JCI119782

Nawashiro, H., Tasaki, K., Ruetzler, C. A., and Hallenbeck, J. M. (1997). TNF-alpha pretreatment induces protective effects against focal cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* 17, 483–490. doi: 10.1097/00004647-199705000-00001

Nijboer, C. H., Heijnen, C. J., Groenendaal, F., May, M. J., van Bel, F., and Kavelaars, A. (2008). A dual role of the NF-kappaB pathway in neonatal hypoxic-ischemic brain damage. *Stroke* 39, 2578–2586. doi: 10.1161/STROKEAHA.108.516401

Nishio, S., Yunoki, M., Chen, Z. F., Anzivino, M. J., and Lee, K. S. (2000). Ischemic tolerance in the rat neocortex following hypothermic preconditioning. *J. Neurosurg.* 93, 845–851. doi: 10.3171/jns.2000.93.5.0845

Nurmi, A. (2004). Nuclear factor-B contributes to infarction after permanent focal ischemia. *Stroke* 35, 987–991. doi: 10.1161/01.STR.0000120732.45951.26

Nurmi, A., Vartiainen, N., Pihlaja, R., Goldsteins, G., Yrjanheikki, J., and Koistinaho, J. (2004). Pyrrolidine dithiocarbamate inhibits translocation of nuclear factor kappa-B in neurons and protects against brain ischaemia with a wide therapeutic time window. *J. Neurochem.* 91, 755–765. doi: 10.1111/j.1471-4159.2004.02756.x

O'Connell, R. M., Chaudhuri, A. A., Rao, D. S., and Baltimore, D. (2009). Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7113–7118. doi: 10.1073/pnas.0902636106

O'Connell, R. M., Rao, D. S., and Baltimore, D. (2012). microRNA regulation of inflammatory responses. *Annu. Rev. Immunol.* 30, 295–312. doi: 10.1146/annurev-immunol-020711-075013

Ohtsuki, T., Ruetzler, C. A., Tasaki, K., and Hallenbeck, J. M. (1996). Interleukin-1 mediates induction of tolerance to global ischemia in gerbil hippocampal CA1 neurons. *J. Cereb. Blood Flow Metab.* 16, 1137–1142. doi: 10.1097/00004647-199611000-00007

O'Neill, L. A., Sheedy, F. J., and McCoy, C. E. (2011). MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nat. Rev. Immunol.* 11, 163–175. doi: 10.1038/nri2957

Ota, A., Ikeda, T., Xia, X. Y., Xia, Y. X., and Ikenoue, T. (2000). Hypoxic-ischemic tolerance induced by hyperthermic pretreatment in newborn rats. *J. Soc. Gynecol. Investig.* 7, 102–105. doi: 10.1016/S1071-5576(00)00038-1

Pacher, P., Bátkai, S., and Kunos, G. (2006). The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol. Rev.* 58, 389–462. doi: 10.1124/pr.58.3.2

Pacher, P., and Haskó, G. (2008). Endocannabinoids and cannabinoid receptors in ischaemia-reperfusion injury and preconditioning. *Br. J. Pharmacol.* 153, 252–262. doi: 10.1038/sj.bjp.0707582

Packard, A. E. B., Hedges, J. C., Bahjat, F. R., Stevens, S. L., Conlin, M. J., Salazar, A. M., et al. (2012). Poly-IC preconditioning protects against cerebral and renal ischemia-reperfusion injury. *J. Cereb. Blood Flow Metab.* 32, 242–247. doi: 10.1038/jcbfm.2011.160

Pahl, H. L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18, 6853–6866. doi: 10.1038/sj.onc.1203239

Panikashvili, D., Mechoulam, R., Beni, S. M., Alexandrovich, A., and Shohami, E. (2005). CB1 cannabinoid receptors are involved in neuroprotection via NF-κB inhibition. *J. Cereb. Blood Flow Metab.* 25, 477–484. doi: 10.1038/sj.jcbfm.9600047

Panikashvili, D., Simeonidou, C., Ben-Shabat, S., Hanus, L., Breuer, A., Mechoulam, R., et al. (2001). An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* 413, 527–531. doi: 10.1038/35097089

Park, M. K., Kang, Y. J., Lee, H. S., Kim, H. J., Seo, H. G., Lee, J. H., et al. (2008). The obligatory role of COX-2 expression for induction of HO-1 in ischemic preconditioned rat brain. *Biochem. Biophys. Res. Commun.* 377, 1191–1194. doi: 10.1016/j.bbrc.2008.10.149

Patel, H., Shaw, S. G., Shi-Wen, X., Abraham, D., Baker, D. M., and Tsui, J. C. S. (2012). Toll-like receptors in ischaemia and its potential role in the pathophysiology of muscle damage in critical limb ischaemia. *Cardiol. Res. Pract.* 2012:121237. doi: 10.1155/2012/121237

Pedersen, B. K. (2011). Exercise-induced myokines and their role in chronic diseases. *Brain Behav. Immun.* 25, 811–816. doi: 10.1016/j.bbi.2011.02.010

Pedersen, C. M., Cruden, N. L., Schmidt, M. R., Lau, C., Bøtker, H. E., Kharbanda, R. K., et al. (2011). Remote ischemic preconditioning prevents systemic platelet activation associated with ischemia-reperfusion injury in humans. *J. Thromb. Haemost.* 9, 404–407. doi: 10.1111/j.1538-7836.2010.04142.x

Peng, Z., Ren, P., Kang, Z., Du, J., Lian, Q., Liu, Y., et al. (2008). Up-regulated HIF-1α is involved in the hypoxic tolerance induced by hyperbaric oxygen preconditioning. *Brain Res.* 1212, 71–78. doi: 10.1016/j.brainres.2008.03.027

Plamondon, H., Blondeau, N., Heurteaux, C., and Lazdunski, M. (1999). Mutually protective actions of kainic acid epileptic preconditioning and sublethal global ischemia on hippocampal neuronal death: involvement of adenosine A1 receptors and K(ATP) channels. *J. Cereb. Blood Flow Metab.* 19, 1296–1308. doi: 10.1097/00004647-199912000-00002

Pradillo, J. M., Fernández-López, D., García-Yébenes, I., Sobrado, M., Hurtado, O., Moro, M. A., et al. (2009). Toll-like receptor 4 is involved in neuroprotection afforded by ischemic preconditioning. *J. Neurochem.* 109, 287–294. doi: 10.1111/j.1471-4159.2009.05972.x

Pradillo, J. M., Romera, C., Hurtado, O., Cárdenas, A., Moro, M. A., Leza, J. C., et al. (2005). TNFR1 upregulation mediates tolerance after brain ischemic preconditioning. *J. Cereb. Blood Flow Metab.* 25, 193–203. doi: 10.1038/sj.jcbfm.9600019

Pun, P. B. L., Lu, J., and Moochhala, S. (2009). Involvement of ROS in BBB dysfunction. *Free Radic. Res.* 43, 348–364. doi: 10.1080/10715760902751902

Quintin, J., Saeed, S., Martens, J. H. A., Giamarellos-Bourboulis, E. J., Ifrim, D. C., Logie, C., et al. (2012). Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* 12, 223–232. doi: 10.1016/j.chom.2012.06.006

Qureshi, I. A., and Mehler, M. F. (2010). Emerging role of epigenetics in stroke: part 1: DNA methylation and chromatin modifications. *Arch. Neurol.* 67, 1316–1322. doi: 10.1001/archneurol.2010.275

Ramirez-Carrozzi, V. R., Nazarian, A. A., Li, C. C., Gore, S. L., Sridharan, R., Imbalzano, A. N., et al. (2006). Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. *Genes Dev.* 20, 282–296. doi: 10.1101/gad.1383206

Ransohoff, R. M., and Brown, M. A. (2012). Innate immunity in the central nervous system. *J. Clin.l Invest.* 122, 1164–1171. doi: 10.1172/JCI58644

Rehni, A. K., and Singh, T. G. (2012). Involvement of CCR-2 chemokine receptor activation in ischemic preconditioning and postconditioning of brain in mice. *Cytokine* 60, 83–89. doi: 10.1016/j.cyto.2012.05.009

Reis, D. J., Berger, S. B., Underwood, M. D., and Khayata, M. (1991). Electrical stimulation of cerebellar fastigial nucleus reduces ischemic infarction elicited by middle cerebral artery occlusion in rat. *J. Cereb. Blood Flow Metab.* 11, 810–818. doi: 10.1038/jcbfm.1991.139

Reis, D. J., Kobylarz, K., Yamamoto, S., and Golanov, E. V. (1998). Brief electrical stimulation of cerebellar fastigial nucleus conditions long-lasting salvage from focal cerebral ischemia: conditioned central neurogenic neuroprotection. *Brain Res.* 780, 161–165. doi: 10.1016/S0006-8993(97)01017-2

Rosenzweig, H. L., Lessov, N. S., Henshall, D. C., Minami, M., Simon, R. P., and Stenzel-Poore, M. P. (2004). Endotoxin preconditioning prevents cellular

inflammatory response during ischemic neuroprotection in mice. *Stroke* 35, 2576–2581. doi: 10.1161/01.STR.0000143450.04438.ae

Rosenzweig, H. L., Minami, M., Lessov, N. S., Coste, S. C., Stevens, S. L., Henshall, D. C., et al. (2007). Endotoxin preconditioning protects against the cytotoxic effects of TNFalpha after stroke: a novel role for TNFalpha in LPS-ischemic tolerance. *J. Cereb. Blood Flow Metab.* 27, 1663–1674. doi: 10.1038/sj.jcbfm.9600464

Rüegger, S., and Großhans, H. (2012). MicroRNA turnover: when, how, and why. *Trends Biochem. Sci.* 37, 436–446. doi: 10.1016/j.tibs.2012.07.002

Schwaninger, M., Schneider, A., Martin-Villalba, A., Weih, F., Vogel, J., and Wirth, T. (1999). NF-kappaB is activated and promotes cell death in focal cerebral ischemia. *Nat. Med.* 5, 554–559. doi: 10.1038/8432

Semple, B. D., Kossmann, T., and Morganti-Kossmann, M. C. (2009). Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. *J. Cereb. Blood Flow Metab.* 30, 459–473. doi: 10.1038/jcbfm.2009.240

Shichita, T., Hasegawa, E., Kimura, A., Morita, R., Sakaguchi, R., Takada, I., et al. (2012). Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. *Nat. Med.* 18, 911–917. doi: 10.1038/nm.2749

Shimizu, M., Saxena, P., Konstantinov, I. E., Cherepanov, V., Cheung, M. M. H., Wearden, P., et al. (2010). Remote ischemic preconditioning decreases adhesion and selectively modifies functional responses of human neutrophils. *J. Surg. Res.* 158, 155–161. doi: 10.1016/j.jss.2008.08.010

Shin, J. A., Park, E.-M., Choi, J.-S., Seo, S.-M., Kang, J. L., Lee, K.-E., et al. (2009). Ischemic preconditioning-induced neuroprotection is associated with differential expression of IL-1beta and IL-1 receptor antagonist in the ischemic cortex. *J. Neuroimmunol.* 217, 14–19. doi: 10.1016/j.jneuroim.2009.06.001

Shpargel, K. B., Jalabi, W., Jin, Y., Dadabayev, A., Penn, M. S., and Trapp, B. D. (2008). Preconditioning paradigms and pathways in the brain. *Cleve. Clin. J. Med.* 75(Suppl. 2), S77–S82. doi: 10.3949/ccjm.75.Suppl_2.S77

Sly, L. M., Rauh, M. J., Kalesnikoff, J., Song, C. H., and Krystal, G. (2004). LPS-Induced Upregulation of SHIP is essential for endotoxin tolerance. *Immunity* 21, 227–239. doi: 10.1016/j.immuni.2004.07.010

Stapels, M., Piper, C., Yang, T., Li, M., Stowell, C., Xiong, Z. G., et al. (2010). Polycomb group proteins as epigenetic mediators of neuroprotection in ischemic tolerance. *Sci. Signal.* 3, ra15–ra15. doi: 10.1126/scisignal.2000502

Stenzel-Poore, M. P., Stevens, S. L., Xiong, Z., Lessov, N. S., Harrington, C. A., Mori, M., et al. (2003). Effect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states. *Lancet* 362, 1028–1037. doi: 10.1016/S0140-6736(03)14412-1

Stephenson, D., Yin, T., Smalstig, E. B., Hsu, M. A., Panetta, J., Little, S., et al. (2000). Transcription factor nuclear factor-kappa B is activated in neurons after focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 20, 592–603. doi: 10.1097/00004647-200003000-00017

Stevens, S. L., Ciesielski, T. M. P., Marsh, B. J., Yang, T., Homen, D. S., Boule, J.-L., et al. (2008). Toll-like receptor 9: a new target of ischemic preconditioning in the brain. *J. Cereb. Blood Flow Metab.* 28, 1040–1047. doi: 10.1038/sj.jcbfm.9600606

Stevens, S. L., Leung, P. Y., Vartanian, K. B., Gopalan, B., Yang, T., Simon, R. P., et al. (2011). Multiple preconditioning paradigms converge on interferon regulatory factor-dependent signaling to promote tolerance to ischemic brain injury. *J. Neurosci.* 31, 8456–8463. doi: 10.1523/JNEUROSCI.0821-11.2011

Stowe, A. M., Wacker, B. K., Cravens, P. D., Perfater, J. L., Li, M. K., Hu, R., et al. (2012). CCL2 upregulation triggers hypoxic preconditioning-induced protection from stroke. *J. Neuroinflammation* 9:33. doi: 10.1186/1742-2094-9-33

Stridh, L., Smith, P. L. P., Naylor, A. S., Wang, X., and Mallard, C. (2011). Regulation of toll-like receptor 1 and -2 in neonatal mice brains after hypoxia-ischemia. *J. Neuroinflammation* 8:45. doi: 10.1186/1742-2094-8-45

Suzuki, H. I., Arase, M., Matsuyama, H., Choi, Y. L., Ueno, T., Mano, H., et al. (2011). MCPIP1 ribonuclease antagonizes dicer and terminates microRNA biogenesis through precursor microRNA degradation. *Mol. Cell* 44, 424–436. doi: 10.1016/j.molcel.2011.09.012

Swigut, T., and Wysocka, J. (2007). H3K27 demethylases, at long last. *Cell* 131, 29–32. doi: 10.1016/j.cell.2007.09.026

Tapuria, N., Kumar, Y., Habib, M. M., Abu-Amara, M., Seifalian, A. M., and Davidson, B. R. (2008). Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury—a review. *J. Surg. Res.* 150, 304–330. doi: 10.1016/j.jss.2007.12.747

Tasaki, K., Ruetzler, C. A., Ohtsuki, T., Martin, D., Nawashiro, H., and Hallenbeck, J. M. (1997). Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. *Brain Res.* 748, 267–270. doi: 10.1016/S0006-8993(96)01383-2

Towfighi, J., Housman, C., Mauger, D., and Vannucci, R. C. (1999). Effect of seizures on cerebral hypoxic-ischemic lesions in immature rats. *Brain Res. Dev. Brain Res.* 113, 83–95. doi: 10.1016/S0165-3806(99)00004-8

Trinchieri, G. (2010). Type I interferon: friend or foe? *J. Exp. Med.* 207, 2053–2063. doi: 10.1084/jem.20101664

Ullrich, O., Merker, K., Timm, J., and Tauber, S. (2007). Immune control by endocannabinoids—new mechanisms of neuroprotection? *J. Neuroimmunol.* 184, 127–135. doi: 10.1016/j.jneuroim.2006.11.018

Vabulas, R. M., Ahmad-Nejad, P., Ghose, S., Kirschning, C. J., Issels, R. D., and Wagner, H. (2002). HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J. Biol. Chem.* 277, 15107–15112. doi: 10.1074/jbc.M111204200

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

van Rooij, E., Sutherland, L. B., Qi, X., Richardson, J. A., Hill, J., and Olson, E. N. (2007). Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* 316, 575–579. doi: 10.1126/science.1139089

Vartanian, K., and Stenzel-Poore, M. (2010). Toll-like receptor tolerance as a mechanism for neuroprotection. *Transl. Stroke Res.* 1, 252–260. doi: 10.1007/s12975-010-0033-5

Vartanian, K. B., Stevens, S. L., Marsh, B. J., Williams-Karnesky, R., Lessov, N. S., and Stenzel-Poore, M. P. (2011). LPS preconditioning redirects TLR signaling following stroke: TRIF-IRF3 plays a seminal role in mediating tolerance to ischemic injury. *J. Neuroinflammation* 8:140. doi: 10.1186/1742-2094-8-140

Veldhuis, W. B., Derksen, J. W., Floris, S., van der Meide, P. H., de Vries, H. E., Schepers, J., et al. (2003). Interferon-beta blocks infiltration of inflammatory cells and reduces infarct volume after ischemic stroke in the rat. *J. Cereb. Blood Flow Metab.* 1029–1039. doi: 10.1097/01.WCB.0000080703.47016.B6

Verstak, B., Hertzog, P., and Mansell, A. (2007). Toll-like receptor signalling and the clinical benefits that lie within. *Inflamm. Res.* 56, 1–10. doi: 10.1007/s00011-007-6093-7

Verstrepen, L., Verhelst, K., van Loo, G., Carpentier, I., Ley, S. C., and Beyaert, R. (2010). Expression, biological activities and mechanisms of action of A20 (TNFAIP3). *Biochem. Pharmacol.* 80, 2009–2020. doi: 10.1016/j.bcp.2010.06.044

Vezzani, A., Moneta, D., Richichi, C., Aliprandi, M., Burrows, S. J., Ravizza, T., et al. (2002). Functional role of inflammatory cytokines and antiinflammatory molecules in seizures and epileptogenesis. *Epilepsia* 43(Suppl. 5), 30–35. doi: 10.1046/j.1528-1157.43.s.5.14.x

Vlasov, T. D., Korzhevskii, D. E., and Polyakova, E. A. (2005). Ischemic preconditioning of the rat brain as a method of endothelial protection from ischemic/repercussion injury. *Neurosci. Behav. Physiol.* 35, 567–572. doi: 10.1007/s11055-005-0095-0

Wacker, B. K., Perfater, J. L., and Gidday, J. M. (2012). Hypoxic preconditioning induces stroke tolerance in mice via a cascading HIF, sphingosine kinase, and CCL2 signaling pathway. *J. Neurochem.* 123, 954–962. doi: 10.1111/jnc.12047

Wang, F., Birch, S. E., He, R., Tawadros, P., Szaszi, K., Kapus, A., et al. (2010). Remote ischemic preconditioning by hindlimb occlusion prevents liver ischemic/reperfusion injury. *Ann. Surg.* 251, 292–299. doi: 10.1097/SLA.0b013e3181bfda8c

Wang, L., Traystman, R. J., and Murphy, S. J. (2008). Inhalational anesthetics as preconditioning agents in ischemic brain. *Curr. Opin. Pharmacol.* 8, 104–110. doi: 10.1016/j.coph.2007.09.005

Wang, Q., Peng, Y., Chen, S., Gou, X., Hu, B., Du, J., et al. (2009). Pretreatment with electroacupuncture induces rapid tolerance to focal cerebral ischemia through regulation of endocannabinoid system. *Stroke* 40, 2157–2164. doi: 10.1161/STROKEAHA.108.541490

Wang, Q., Xiong, L., Chen, S., Liu, Y., and Zhu, X. (2005). Rapid tolerance to focal cerebral ischemia in rats is induced by preconditioning with electroacupuncture: window of protection and the role of adenosine. *Neurosci. Lett.* 381, 158–162. doi: 10.1016/j.neulet.2005.02.019

Wang, R. Y., Yang, Y. R., and Yu, S. M. (2001). Protective effects of treadmill training on infarction in rats. *Brain Res.* 922, 140–143. doi: 10.1016/S0006-8993(01)03154-7

Wang, X., Li, X., Currie, R. W., Willette, R. N., Barone, F. C., and Feuerstein, G. Z. (2000). Application of real-time polymerase chain reaction to quantitate induced expression of interleukin-1beta mRNA in ischemic brain tolerance. *J. Neurosci. Res.* 59, 238–246. doi: 10.1016/S1385-299X(00)00015-5

Wang, Y., Wang, Y., and Yang, G.-Y. (2013). MicroRNAs in cerebral ischemia. *Stroke Res. Treat.* 2013:276540. doi: 10.1155/2013/276540

Wang, Y.-C., Lin, S., and Yang, Q.-W. (2011). Toll-like receptors in cerebral ischemic inflammatory injury. *J. Neuroinflammation* 8:134. doi: 10.1186/1742-2094-8-134

Wu, T.-C., and Grotta, J. C. (2013). Hypothermia for acute ischaemic stroke. *Lancet Neurol.* 12, 275–284. doi: 10.1016/S1474-4422(13)70013-9

Yanamoto, H., Hashimoto, N., Nagata, I., and Kikuchi, H. (1998). Infarct tolerance against temporary focal ischemia following spreading depression in rat brain. *Brain Res.* 784, 239–249. doi: 10.1016/S0006-8993(97)01344-9

Yang, Q.-W., Lu, F.-L., Zhou, Y., Wang, L., Zhong, Q., Lin, S., et al. (2011). HMBG1 mediates ischemia-reperfusion injury by TRIF-adaptor independent Toll-like receptor 4 signaling. *J. Cereb. Blood Flow Metab.* 31, 593–605. doi: 10.1038/jcbfm.2010.129

Yin, W., Signore, A. P., Iwai, M., Cao, G., Gao, Y., Johnnides, M. J., et al. (2007). Preconditioning suppresses inflammation in neonatal hypoxic ischemia via Akt activation. *Stroke* 38, 1017–1024. doi: 10.1161/01.STR.0000258102.18836.ca

Yoshimura, A., Naka, T., and Kubo, M. (2007). SOCS proteins, cytokine signalling and immune regulation. *Nat. Rev. Immunol.* 7, 454–465. doi: 10.1038/nri2093

Yu, J. T., Lee, C. H., Yoo, K.-Y., Choi, J. H., Li, H., Park, O. K., et al. (2010). Maintenance of anti-inflammatory cytokines and reduction of glial activation in the ischemic hippocampal CA1 region preconditioned with lipopolysaccharide. *J. Neurol. Sci.* 296, 69–78. doi: 10.1016/j.jns.2010.06.004

Yunoki, M., Nishio, S., Ukita, N., Anzivino, M. J., and Lee, K. S. (2003). Hypothermic preconditioning induces rapid tolerance to focal ischemic injury in the rat. *Exp. Neurol.* 181, 291–300. doi: 10.1016/S0014-4886(03)00056-6

Zhang, F., Wu, Y., and Jia, J. (2011). Exercise preconditioning and brain ischemic tolerance. *Neuroscience* 177, 170–176. doi: 10.1016/j.neuroscience.2011.01.018

Zhang, M., Adler, M. W., Abood, M. E., Ganea, D., Jallo, J., and Tuma, R. F. (2009). CB2 receptor activation attenuates microcirculatory dysfunction during cerebral ischemic/reperfusion injury. *Microvasc. Res.* 78, 86–94. doi: 10.1016/j.mvr.2009.03.005

Zhang, M., Martin, B. R., Adler, M. W., Razdan, R. K., Jallo, J. I., and Tuma, R. F. (2007). Cannabinoid CB2 receptor activation decreases cerebral infarction in a mouse focal ischemia/reperfusion model. *J. Cereb. Blood Flow Metab.* 27, 1387–1396. doi: 10.1038/sj.jcbfm.9600447

Zhao, P., and Zuo, Z. (2004). Isoflurane preconditioning induces neuroprotection that is inducible nitric oxide synthase-dependent in neonatal rats. *Anesthesiology* 101, 695–703. doi: 10.1097/00000542-200409000-00018

Zhou, P., Qian, L., D'Aurelio, M., Cho, S., Wang, G., Manfredi, G., et al. (2012). Prohibitin reduces mitochondrial free radical production and protects brain cells from different injury modalities. *J. Neurosci.* 32, 583–592. doi: 10.1523/JNEUROSCI.2849-11.2012

Zhou, P., Qian, L., Zhou, T., and Iadecola, C. (2005). Mitochondria are involved in the neurogenic neuroprotection conferred by stimulation of cerebellar fastigial nucleus. *J. Neurochem.* 95, 221–229. doi: 10.1111/j.1471-4159.2005.03358.x

Zhuang, S.-Y., Bridges, D., Grigorenko, E., McCloud, S., Boon, A., Hampson, R. E., et al. (2005). Cannabinoids produce neuroprotection by reducing intracellular calcium release from ryanodine-sensitive stores. *Neuropharmacology* 48, 1086–1096. doi: 10.1016/j.neuropharm.2005.01.005

Zimmermann, C., Ginis, I., Furuya, K., Klimanis, D., Ruetzler, C., Spatz, M., et al. (2001). Lipopolysaccharide-induced ischemic tolerance is associated with increased levels of ceramide in brain and in plasma. *Brain Res.* 895, 59–65. doi: 10.1016/S0006-8993(01)02028-5

Zwagerman, N., Plumlee, C., Guthikonda, M., and Ding, Y. (2010). Toll-like receptor-4 and cytokine cascade in stroke after exercise. *Neurol. Res.* 32, 123–126. doi: 10.1179/016164109X12464612122812

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 September 2013; accepted: 17 February 2014; published online: 04 March 2014.

Citation: Garcia-Bonilla L, Benakis C, Moore J, Iadecola C and Anrather J (2014) Immune mechanisms in cerebral ischemic tolerance. Front. Neurosci. ***8****:44. doi: 10.3389/fnins.2014.00044*

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in
NEUROSCIENCE

REVIEW ARTICLE
published: 14 February 2014
doi: 10.3389/fnins.2014.00023

Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis

Bakri M. Assas[1,2]*, Joanne I. Pennock[1†] and Jaleel A. Miyan[3†]

[1] *Translational Medicine, Faculty of Medical and Human Sciences, The University of Manchester, Manchester, UK*
[2] *Department of Immunology, Faculty of Applied Sciences, King Abdulaziz University, Jeddah, Saudi Arabia*
[3] *Neurosciences, Faculty of Life Sciences, The University of Manchester, Manchester, UK*

Edited by:
Adam Denes, University of Manchester, UK

Reviewed by:
Krisztina J. Kovacs, Institute of Experimental Medicine, Hungary
Cynthia Detre, Independent Scientist, USA

****Correspondence:***
Bakri M. Assas, Translational Medicine, Faculty of Medical and Human Sciences, The University of Manchester, AV Hill Building, Oxford Road, Manchester M13 9PT, UK
e-mail: mushref.assas@manchester.ac.uk
† Joint last authors.

The question of how the neural and immune systems interact in host defense is important, integrating a system that senses the whole body with one that protects. Understanding the mechanisms and routes of control could produce novel and powerful ways of promoting and enhancing normal functions as well as preventing or treating abnormal functions. Fragmentation of biological research into specialities has resulted in some failures in recognizing and understanding interactions across different systems and this is most striking across immunology, hematology, and neuroscience. This reductionist approach does not allow understanding of the *in vivo* orchestrated response generated through integration of all systems. However, many factors make the understanding of multisystem cross-talk in response to a threat difficult, for instance the nervous and immune systems share communication molecules and receptors for a wide range of physiological signals. But, it is clear that physical, hard-wired connections exist between the two systems, with the key link involving sensory, unmyelinated nerve fibers (c fibers) containing the neuropeptide calcitonin gene-related peptide (CGRP), and modified macrophages, mast cells and other immune and host defense cells in various locations throughout the body. In this review we will therefore focus on the induction of CGRP and its key role in the neuroimmune axis.

Keywords: CGRP, TRPV1, neuroimmunology, TNF-alpha, C fibers, gut-brain axis

SENSORY NEUROTRANSMITTER CGRP

Sensory neurotransmitters have been extensively studied and their ability to affect different body functions has been shown in a range of studies. One of the main sensory neurotransmitters involved in immune function is CGRP. CGRP exemplifies a neuroimmune connector, since it is released at the site of stimulation, affecting immediate responses as well as mediating information flow to the rest of the nervous system. CGRP is a critical, highly expressed sensory signal, making it an important member of neuro-immune communication pathways. C fibers, the smallest diameter unmyelinated sensory neurons, are the main source of this neuropeptide. Their small diameter generates one of the lowest threshold response elements in the nervous system indicating their vital role. To date, this low threshold has placed them in the category of nociceptive neurons as they are the first to register damage/toxins through the pain pathway. This categorization is reinforced by the fact that c fibers express on their surface the transient receptor potential vanilloid 1 (TRPV1) which is a key responder to tissue damage. However, below the pain threshold, C fibers are likely to be playing a critical role in physiological systems and in particular, in host monitoring and activation of host defense and immune responses due to their low activation potential.

CGRP RELEASE *IN VIVO*

CGRP is released in response to activation of TRPV1 in both the nervous and immune systems. In the nervous system, TRPV1 is expressed along the entire length of the sensory c fiber neurons, from the periphery to the somata in the CNS (Szallasi, 1995). These neurons innervate every organ and tissue in the body (Buck and Burks, 1986; Holzer, 1991; Szallasi and Blumberg, 1999). Although a key exogenous ligand for TRPV1 is capsaicin, TRPV1 is also activated by a range of other endogenous agonists including heat (>43°C) (Caterina et al., 1997; Tominaga et al., 1998), protons (∼pH 4.5) (Vyklicky et al., 1998; Jordt et al., 2000), lipids like anandamide (Olah et al., 2001), phosphatidylinositol(4,5)-biphosphate (PIP_2) (Chuang et al., 2001), and voltage (Gunthorpe et al., 2000) (summarized in **Figure 1**). Heat and low pH activate TRPV1 by distinct molecular recognition sites (Jordt et al., 2000). Additionally, TRPV1 has been suggested to be a mechanosensitive receptor in mediating nociceptive signals; for example TRPV1 deficient mice exhibit reduced sensitivity to post colorectal distension compared to controls (Jones et al., 2005) adding an interesting physiological element to TRPV1 activation. In rats, sensory vanilloid receptor-bearing nerves are classed as peptidergic and/or purinergic (Guo et al., 1999). Peptidergic nerves express TRPV1 and co-express neuropeptides whilst purinergic nerves express TRPV1 and co-express an adenosine triphosphate (ATP) gated ion-channel known as $P2X_3$ (Yiangou et al., 2001).

Besides the well documented role of TRPV1 in the nervous system, we now know that it is also expressed on immune cells, which has direct relevance to this review. Absence of TRPV1 signaling has been shown variously to exacerbate inflammation (Massa

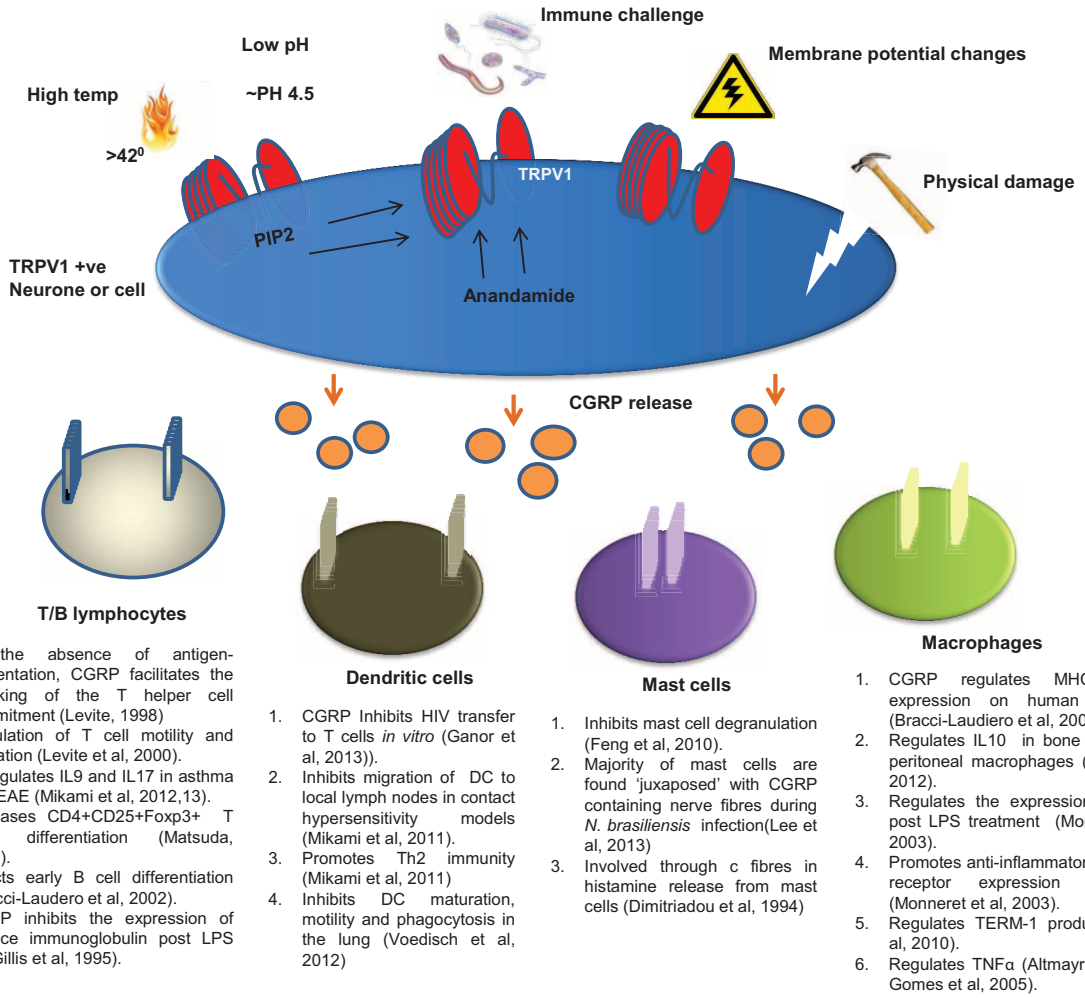


FIGURE 1 | Pathways of CGRP release via TRPV1 activation. TRPV1 is activated by many external factors associated with pain but also regular sensation below pain thresholds. These include the well characterized responses to heat, pH, and damage, as well as to toxins, immune challenges and membrane potential changes brought about by physiological and pathological processes. Endogenous and/or intracellular activation can also occur through the actions of PIP2 and Anandomide. Any activation of TRPV1 results in release of CGRP from the neuron. This released CGRP then acts on its specific receptor on cells immediately adjacent to the neuron, which in the case of sensory c fibers can be lymphocytes, DCs, mast cells, macrophages or cells within the hemopoietic microenvironment of the bone marrow (not shown). The range of activities stimulated by CGRP are listed below each cell type.

et al., 2006; Huang et al., 2009) and these effects can be attributed indirectly to the subsequent lack of CGRP. Similarly, agonism of TRPV1 with capsaicin has been shown to inhibit differentiation and cytokine secretion of human dendritic cells (Toth et al., 2009), thereby reducing early activation signals on challenge.

There are two isoforms of CGRP known: αCGRP and βCGRP. αCGRP has been shown to be a potent vasodilator and exhibits marked synergism with inflammatory mediators to induce local edema (Bernard and Shih, 1990). αCGRP's receptors are found on mouse bone marrow cells, providing strong evidence toward an eminent effect for αCGRP in modulating immune cell function and differentiation which in turn may affect the inflammatory cascade in response to a pathological threat (Mullins et al., 1993). αCGRP has also been identified as the predominant isotype in sensory neurons in the dorsal root ganglion (DRG) (Gibson et al., 1988). Unless stated otherwise, the observations discussed below refer to αCGRP only.

PHYSIOLOGICAL ROLE FOR CGRP

CGRP was first described as a vasodilator nearly 30 years ago (Brain et al., 1985). It is now well-established that CGRP regulates blood pressure (Brain and Grant, 2004), is associated with the onset of migraines when at high levels (Olesen, 2011a,b), promotes the maintenance of mucosal integrity in the gastrointestinal tract (GIT) (Szolcsanyi and Bartho, 2001), aids stem cell growth and maintenance *in vitro* (Dong et al., 2010) and modulates keratinocyte growth (Roggenkamp et al., 2013). CGRP plays a role in facilitating the absorption of intraluminal amino acids across the distal parts of the intestines (Mourad et al., 2009). In the lungs and elsewhere, CGRP promotes wound healing (Zhou et al., 2013). In the stomach, gastric acid stimulates the release of

CGRP via activation of local afferent neurons. CGRP itself orchestrates the secretion of chemical factors that inhibit gastric acid secretion and induce muscle repair (Aihara et al., 2005, 2006). Furthermore, CGRP and other neuropeptide containing nerve fibers extend all the way to the epithelial cell lining of the gut, suggesting a role in monitoring of the external (gut) environment. An association with immune cells present in gut epithelium also suggests an important role in the immune responses generated there (Engel et al., 2010, 2011).

CGRP AND IMMUNITY

CGRP is the main neurotransmitter of the nociceptive sensory c fibers, but is also present in other sensory nerve fibers i.e., type A and B medium-sized neurons. CGRP is released when these nerves are activated by specific agonists of TRPV1 usually during trauma or injury and therefore additionally associated with pain perception. However, c fibers have also been implicated in non-nociceptive activities including modulation of immune responses, through neuropeptide release (Beresford et al., 2004; Shepherd et al., 2005a,b). In fact CGRP is abundant in many immune organs. Nerve fibers containing CGRP have been identified in bone marrow, thymus, spleen, lymph nodes, skin, lungs, and gut and CGRP receptors are found on many hematopoietic cell types (Santambrogio et al., 1993; Petitto et al., 1994; Mach et al., 2002). In the bone marrow, these fibers accompany noradrenergic sympathetic fibers and are distributed throughout the marrow. The role of sensory neurotransmitters in the hematopoietic process has been examined for CGRP. Treatment with capsaicin, a potent neurotoxin to c fibers and activator of TRPV1, produced a dramatic change in bone marrow hematopoiesis when measured by *in vitro* colony-forming assays suggesting that CGRP has direct access to hematopoietic progenitors (Broome et al., 2000). CGRP positive nerves have also been detected in the cortical and vascular regions of lymph nodes. No evidence has been obtained/presented for the presence of parasympathetic nerves in lymph nodes or for the presence of the parasympathetic neurotransmitter acetyl choline (Schafer et al., 1998). However, non-neural sources for acetyl choline in lymphatic organs have been identified (Rosas-Ballina et al., 2011) where it is secreted by resident memory T cells in the spleen facilitating vagal anti-inflammatory functions, therefore, playing part in the vagal-immune pathways.

Many studies have demonstrated specific roles for CGRP and other peptides, including substance P, in the generation of both pro and anti-inflammatory immune responses. CGRP in particular has been the focus of many studies trying to unveil its involvement in a range of inflammatory models and immune conditions e.g., diabetes (Morrison et al., 2009), sepsis (De Winter et al., 2009), EAE (Mikami et al., 2012a) Crohn's Disease (Smith and Smid, 2005), and ulcerative colitis (Engel et al., 2012; Li et al., 2013a). In rats, dextran sulfate sodium (DSS)-induced colitis worsened when treated with a CGRP antagonist suggesting a protective role for CGRP in colitis (Engel et al., 2010).

Once released, CGRP plays multifunctional roles at different sites by binding to its receptor calcitonin receptor like receptor (CRLR) and its receptor activity-modifying protein 1 (RAMP1) found on T and B lymphocytes (Mikami et al., 2012a,b), macrophages (Fernandez et al., 2001), mast cells (Eftekhari et al., 2013), and dendritic cells (Mikami et al., 2011) among others (See **Figure 1**). CGRP is released from afferent fibers at the site of stimulation, presenting a motor-like mode of action conducted in parallel with afferent signaling to the DRG (Holzer and Maggi, 1998). However, it is well documented that cells of the immune system, i.e., lymphocytes (Wang et al., 1999), monocytes (Bracci-Laudiero et al., 2005), and macrophages (Linscheid et al., 2004; Lee and Zhang, 2012) also synthesize CGRP. It is thought that CGRP operates through the intracellular molecules Protein kinase C (PKC), PKA, and MAP kinase (MAPK) (Li et al., 2013b; Zhou et al., 2013) particularly in the lung and uterus (Bai et al., 2012; Mikami et al., 2013; Wong et al., 2013). In addition, CGRP influences NF-kappa-B activation and induction of cAMP suppressor genes in immune cells, particularly dendritic cells (Harzenetter et al., 2007) thereby regulating function.

CGRP REGULATES DENDRITIC CELL FUNCTIONS

Physiologically, CGRP was first found to be closely associated with dendritic cells (DC) in the skin (Hosoi et al., 1993). This study was the first to show a physical association between CGRP and Langerhans cells (LC) in the human epidermis. Functionally, it was one of the earliest publications to suggest an inhibitory function for CGRP on LC antigen presentation. So far, the majority of publications on the role of CGRP on DC suggest a predominant anti-inflammatory rather than pro-inflammatory effect. CGRP can modulate antigen presentation in DC (Mikami et al., 2011), and inhibit lipopolysaccharide induction of co-stimulatory signaling via the CD80 and CD28 receptors on dendritic cells and monocytes thereby affecting the function of T cells (Fox et al., 1997). Recently, Ganor et al. published a study on the role of CGRP during HIV-1 infection. Initially, CGRP plasma levels increased during the viral infection interfering with the induction of infection through LCs. CGRP inhibited the HIV-1 transfer to T cells, dampening the viral replication by 6 fold (Ganor et al., 2013). CGRP has also been shown to inhibit the migration of LC to local lymph nodes in contact hypersensitivity models, promoting Th2 type immunity in the process (Mikami et al., 2011). *In vitro* lung (DC) maturation was inhibited with CGRP (Rochlitzer et al., 2011). This correlated with a down regulation of the CGRP receptor on these DC during airway inflammation and these effects were reversed with the CGRP receptor antagonist $hCGRP_{8-37}$. CGRP continued to influence the antigen presenting functions of DCs in the lungs even when DCs were pre-treated with CGRP. DC phagocytosis and motility were modulated by CGRP (Voedisch et al., 2012) suggesting an important role for CGRP in maintaining immune integrity in the lungs via the control of DC functions. Similar to its effects on DCs in the skin and lungs, CGRP also inhibits the function of bone marrow-derived DCs. Through its receptor complex, CGRP inhibited the production of (Tumor necrosis factor) TNFα and IL12 post-LPS treatment highlighting its potent anti-inflammatory roles (Tsujikawa et al., 2007). CGRP has additionally been identified as a potent inhibitor of TLR induced inflammatory agents TNFα and CCL4 (Harzenetter et al., 2007). CGRP has also been shown to down regulate the surface expression of HLA-DR and CD86 on mature and immature human DC (Carucci et al., 2000), suggesting a mechanism whereby CGRP may dampen DC-T cell interaction.

CGRP REGULATES LYMPHOCYTE DIFFERENTIATION AND CYTOKINE PRODUCTION

CGRP shares aclose relationship with T cells. The interaction of T cell with DCs and macrophages, both of which express the CGRP receptor, is influenced by CGRP expression. (Ben-Horin and Chowers, 2008; Rochlitzer et al., 2011; Jusek et al., 2012; Holzmann, 2013). The capability of CGRP to regulate T cell function was proposed as early as 1988 (Umeda et al., 1988). In the last decade, studies have demonstrated a Th2 polarized T cell response after CGRP administration. Publications strongly suggest a TH2 preference by CGRP, through its influence on T cell differentiation (Levite, 2000; Ding et al., 2008). In more than one study, CGRP exposure to different DCs enhanced a TH2 type immunity increasing IL4 production while decreasing the TH1-associated cytokines interferon gamma (IFNγ) and IL2 (Wang et al., 1992; Tokoyoda et al., 2004; Mikami et al., 2011). The observed sensitization, according to Tokoyoda et al., occurs through the CGRP receptor activating cAMP/PKA pathways with the involvement of the conventional CD3/CD28 co-stimulation signal in T cells (Tokoyoda et al., 2004). CGRP has been shown to promote TH2 cytokine production *in vitro* and decrease TH1 cytokines (Levite, 1998, 2008). In 1998 and again in 2001, Levite et al., compared antigen-driven and neuropeptide-driven cytokine secretion from T helper cells (Levite, 1998; Levite and Chowers, 2001). They found that in a controlled *in vitro* environment TH0, TH1, and TH2 cells all produced "unconventional" cytokines respective to their phenotype when exposed to neuropeptide, with TH1 secreting IL4, TH2 secreting IFNγ, and TH0 secreting both. This suggests an ability to break the T helper line commitment and regulation of T cell functions. CGRP also up regulated IL9 and IL17 in both asthma and experimental autoimmune encephalomyelitis (EAE) models (Mikami et al., 2012a, 2013). IL17 has been attributed to a range of inflammatory functions and roles; however, its association with CGRP is still not understood. In an EAE model, IL17 levels dropped in RAMP-$1^{-/-}$ mice and TH17 functions were suppressed, suggesting a role for CGRP in the TH17 induced EAE (Mikami et al., 2012a). This has since been corroborated in models of psoriasis where TH17 cells are the dominant inflammatory phenotype (Ostrowski et al., 2011). In contrast, the association of CGRP with CD4+CD25+Foxp3+ cells has also been studied in a model of EAE (Matsuda et al., 2012). CGRP transfected DCs were able to increase differentiation of CD4+CD25+Foxp3+ regulatory cells (Matsuda et al., 2012). CGRP, is also produced by B lymphocytes in inflammatory conditions under the influence of nerve growth factor (NGF) (Bracci-Laudiero et al., 2002). The effect of CGRP on early B cell differentiation has been examined using a pre-B cell line population (70Z/3). CGRP inhibited the expression of surface immunoglobulin in response to LPS (McGillis et al., 1993, 1995). These studies suggest a crucial role for CGRP in early B cell differentiation, a finding supported by later studies (Fernandez et al., 2000; Schlomer et al., 2007).

CGRP AND IMMUNE MOTILITY, MIGRATION, AND ADHESION

In 1992, CGRP was described as a neuropeptide with "chemotactic" properties on human CD4 and CD8 T lymphocytes in the skin, inducing T cell trafficking (Foster et al., 1992). Ever since, T cells more than any other cell, have been the main targets for studies to understand the effect of CGRP on immune cell migration, adhesion, and motility. CGRP plays a key role in T cell adhesion to fibronectin (Levite, 1998; Levite et al., 1998) and beta integrin mediated T cell migration. Somatostatin, CGRP and neuropeptide Y all induced of freshly purified T cells to fibronectin coated plates (Springer et al., 2003). Deletion of CGRP results in a sustained decrease in leukocyte circulation and migration, highlighting its role in the bone marrow and in the mobilization of immune cells (Broome and Miyan, 2000; Broome et al., 2000). In the gut CGRP from c fibers stimulates T cell migration (Talme et al., 2008). *In vitro* studies showed that in contrast to other neuropeptides, CGRP can stimulate the migration of CD3 T cells into collagen matrix (Talme et al., 2008), an effect inhibited with CGRP receptor antagonist. In the absence of other immunological signals extracellular K(+) plays a critical role in stimulating T cell-integrin induced adhesion and migration. T cell voltage-gated K channels (Kv1.3) are targeted by a number of molecules to control T cell-integrin induced motility (Levite et al., 2000). CGRP binding to T cells, through its receptor, opens the voltage-gated K(+) channels, releasing K(+) from the intracellular matrix and activating β1 integrin. This facilitates T cell integrin-induced function highlighting a critical role for CGRP in alternative pathways for T cell adhesion, migration and motility. The effect of CGRP on macrophage and monocyte motility and chemotactic activity has also been studied. *In vitro*, inoculated promastigotes from cutaneous *Leishmania major,* demonstrated that CGRP, substance P, and somatstatin had regulatory effects on macrophage chemotactic activity (Ahmed et al., 1998; Han et al., 2010). CGRP was suggested to play an important role in human monocyte adhesion and migration (Linscheid et al., 2004). Human neutrophils cultured *in vitro* with CGRP and LPS secreted high levels of the chemokine IL8 (He et al., 2002), whilst CGRP has also been proposed to stimulate eosinophil migration (Dunzendorfer et al., 1998). CGRP increased the adhesion-related DC CD103+ ligand epithelial cadherin (E-cadherin) expression in human bronchial epithelial cells (Bai et al., 2012).

REGULATION OF OTHER IMMUNE CELLS

A wealth of literature can be found on the role of CGRP in the inhibition of mast cell degranulation. Many studies have reported mast cells and CGRP fibers "juxtaposed" in different models (Bienenstock et al., 1987; Lee et al., 2013). Controlled low dose capsaicin has been used to induce the release of CGRP from sensory nerves (Demirbilek et al., 2004). It has been estimated that nearly 60–70% of mast cells in the jejunum of rats infected with *Nippostrongylus brasiliensis* are juxtaposed to CGRP containing nerve fibers suggesting a role for this association in the immune response to this infection at least (Bienenstock et al., 1987; Dimitriadou et al., 1994) but no doubt in others also. Moreover, desensitization or deletion of c fibers with high doses of capsaicin severely impaired mast cell recruitment during *Schistosoma mansoni* infection in mice (De Jonge et al., 2003; Van Nassauw et al., 2007). Similarly, CGRP inhibited mast cell degranulation in a model of cerebral ischemia in rats (Feng et al., 2010) and in response to anti/IgE treatments, in comparison to other

neuropeptides, in an *in vitro* assay with primary human mast cells (Kulka et al., 2008).

CGRP receptor is also expressed on human monocytes (Caviedes-Bucheli et al., 2008). Under the influence of NGF, CGRP has been shown to regulate the expression of MHCII, CD86 and the production of IL10 in human monocytes *in vitro* (Bracci-Laudiero et al., 2005). However, it seems that the role of CGRP in monocytes/macrophages seems to be less direct than that seen in other immune cells. CGRP is synthesized by monocytes/macrophages and can regulate immune functions of these cells (Ma et al., 2010). For instance CGRP can regulate CD11b expression after LPS exposure in human macrophages and neutrophils promoting anti-inflammatory functions (Monneret et al., 2003). Recently, macrophages have been studied in the context of pain induction and their involvement in pain pathways during inflammation (Hasegawa-Moriyama et al., 2013; Isami et al., 2013; Taves et al., 2013). In various studies, CGRP treatment increased IL10 from both peritoneal and bone marrow derived macrophages in response to LPS (Jusek et al., 2012). CGRP receptor absence resulted in the influx of neutrophils and inflammatory mediators into the peritoneal cavity (Jusek et al., 2012). CGRP was able to regulate the synthesis of triggering receptor expressed on myeloid cells-1 (TERM-1) in LPS-induced macrophages (Li et al., 2010). This effect was absent without LPS. However, the main contribution of CGRP to macrophage function is likely to incorporate its newly found links to the pro-inflammatory cytokine TNFα. This is the main cytokine released by macrophages in response to pathological challenges.

CGRP AND TNFα: A NEW CHAPTER

TNFα and CGRP are two important molecules that demonstrate a key bi-directional influence between the nervous and immune systems. TNFα functions through its receptors TNFRI and TNFRII. These receptors are found on glial cells in the spinal cord which, when activated, release prostaglandins, activating in turn nociceptive neurons and eliciting a pain response. Thus, the involvement of TNFα in peripheral and central pain is no longer a matter of doubt (Saito et al., 2010; Zhang et al., 2010a; Bressan et al., 2011; Gim et al., 2011; Teodorczyk-Injeyan et al., 2011). TNFα has been associated with the enhancement of trigeminal neuronal sensitivity to capsaicin in rats (Khan et al., 2008); exposure to TNFα for 5 min doubles the sensitivity of fura-loaded trigeminal neurons to capsaicin via TRPV1. Both TNFα receptors were present on capsaicin-sensitive trigeminal neurons co-expressed with TRPV1 (Khan et al., 2008). The presence of the TNFα receptors next to TRPV1 suggests that TNFα sensitizes TRPV1 positive neurons through its receptors. TNFRI plays a role in sensitizing tetrodotoxin (TTX)-resistant sodium channels (Jin and Gereau, 2006). It is likely that TNFRI has additional functions in immediate hyperalgesia and nociception to damage (Sommer et al., 1998), whileTNFRII is thought to have a role in nociception in persistent/chronic injurious conditions (Constantin et al., 2008; Schafers et al., 2008). A recent study has also linked TNFα to meningeal nociception (Zhang et al., 2010b), where it was suggested that TNFα functioned through TNFRI & TNFRII on nociceptive neurons and was associated with the "throbbing" sensation significant in migraines. Interestingly, neutralizing antibodies against TNFRI modulated thermal and mechanical hyperalgesia while antibodies against TNFRII had no affect (Sommer et al., 1998). Electrophysiological techniques showed that the local application of TNFα at the peripheral level of sensory neurons excites TRPV1 bearing sensory c fibers suggesting a specific role for TNFα in nociception (Sorkin and Doom, 2000). Additionally, TNFα increased CGRP release from sensitized capsaicin sensitive neurons when given 5 min prior to capsaicin treatment in comparison to vehicle treated rats (Khan et al., 2008). During LPS endotoxin treatment the increase in CGRP release was attributed to TNFα sensitizing afferent neurons in the presence of the prostaglandin sub types, prostanoids (Hua et al., 1996). These studies suggest that the nociceptive functions of TNFα occur via the specific capsaicin receptor TRPV1.

The activation of the specific capsaicin receptor TRPV1 located on c fibers or capsaicin sensitive neurons results in the release of sensory neuropeptides i.e., CGRP and SP (Helliwell et al., 1998; Bhave et al., 2002; Shepherd et al., 2005a; Ren et al., 2011). Additionally, reduction in CGRP tissue expression levels was seen in the dorsal root ganglia of rats injected with Entanercept (anti-TNFα treatment) (Horii et al., 2010). In both rats and humans with rheumatoid arthritis, CGRP and SP levels were reduced by 50% in the serum after treating with Etanercept (Origuchi et al., 2010). By contrast, TRPV1 activation via capsaicin and SA13353 (capsaicin analog) attenuated LPS-induced TNFα release in serum, and this effect was partially inhibited with CGRP antagonist treatment and in TRPV1$^{-/-}$ or sensory denervated mice (Tsuji et al., 2009) suggesting a regulatory function for CGRP on TNFα i.e., CGRP is able to downregulate TNFα *in vivo*. Both small doses (1 mg/kg) and large doses (150 mg/kg) of the TRPV1 agonist capsaicin have been found to downregulate serum levels of a range of pro-inflammatory cytokines including TNFα (Demirbilek et al., 2004), and the effects of capsaicin are attenuated in the presence of the antagonist for either TRPV1 or CGRP (Peng and Li, 2009).

In Toll-like receptor stimulated dendritic cells, CGRP causes a rapid up-regulation of inducible cAMP early repressor (ICER) which competes with ATF-2 for binding at the *Tnf*α promoter gene preventing gene expression of TNFα (Altmayr et al., 2010). In experiments using a non-irritating capsaicin analog (Vanillyl nonanoate) to examine the protective role capsaicin has on gastric mucosa (Luo et al., 2011), increased release of CGRP via TRPV1 activation had the ability to attenuate TNFα release in the serum post ethanol treatment. Blocking CGRP release from TRPV1 caused an increase in TNFα serum levels and increased gastric mucosal damage (Luo et al., 2011). Taken together all the findings suggest the presence of a TNFα-CGRP regulatory "loop" that is mediated via TRPV1 bearing sensory neurons releasing CGRP and/or through intracellular transcriptional interference of TNFα expression by immune cells.

CONCLUSION

CGRP has critical and wide-ranging control functions in various body systems and is particularly critical to normal hemopoietic and immune functions. CGRP-containing c nerve fibers are found everywhere in the body and are associated, in many locations, with specific immune cells including dendritic cells, mast

cells and T cells. It appears to be the key mediator of neuro-immune communication with the c fibers acting as both sensory pathways, informing the nervous system of peripheral challenges, and as a local controller of immune functions. Knocking out this neural system results in major down-regulation of bone marrow hemopoietic output supporting a role for these nerve fibers in sensory feedback to immune control centers, already identified in the central nervous system by Denes et al. (2005). It is therefore becoming increasingly clear that this intimate connection between a specific subset of specialist sensory neurons and immune cells throughout the body, together with the connections to the brain and host defense control centers, is vital to the integrated, coordinated host defense response to any peripheral challenge. Moreover, it is clear that immune mediators, specifically pro-inflammatory cytokines IL-1a and TNFα, have direct effects on brain activity and whole body responses through the stimulation of specific circuits involved in so-called sickness behaviors (Dantzer and Kelley, 2007) that channel energy into host defense at the cost of social and other activities. A more complete understanding of this neural-immune interaction is vital to uncover key avenues for intervention to override abnormal functions and improve normal functions.

REFERENCES

Ahmed, A. A., Wahbi, A., Nordlind, K., Kharazmi, A., Sundqvist, K. G., Mutt, V., et al. (1998). *In vitro* Leishmania major promastigote-induced macrophage migration is modulated by sensory and autonomic neuropeptides. *Scand. J. Immunol.* 48, 79–85. doi: 10.1046/j.1365-3083.1998.00380.x

Aihara, E., Hayashi, M., Sasaki, Y., Kobata, A., and Takeuchi, K. (2005). Mechanisms underlying capsaicin-stimulated secretion in the stomach: comparison with mucosal acidification. *J. Pharmacol. Exp. Ther.* 315, 423–432. doi: 10.1124/jpet.105.087619

Aihara, E., Sasaki, Y., Ise, F., Kita, K., Nomura, Y., and Takeuchi, K. (2006). Distinct mechanisms of acid-induced HCO3- secretion in normal and slightly permeable stomachs. *Am. J. Physiol. Gastrointest. Liver Physiol.* 291, G464–G471. doi: 10.1152/ajpgi.00048.2006

Altmayr, F., Jusek, G., and Holzmann, B. (2010). The neuropeptide calcitonin gene-related peptide causes repression of tumor necrosis factor-alpha transcription and suppression of ATF-2 promoter recruitment in Toll-like receptor-stimulated dendritic cells. *J. Biol. Chem.* 285, 3525–3531. doi: 10.1074/jbc.M109.066787

Bai, H. B., Lu, Y. P., Duan, J. X., Zhou, Y., Sun, G. Y., and Guan, C. X. (2012). [Effects of CGRP on the E-cadherin expression in human bronchial epithelial cells]. *Zhongguo Ying Yong Sheng Li Xue Za Zhi* 28, 346–349.

Ben-Horin, S., and Chowers, Y. (2008). Neuroimmunology of the gut: physiology, pathology, and pharmacology. *Curr. Opin. Pharmacol.* 8, 490–495. doi: 10.1016/j.coph.2008.07.010

Beresford, L., Orange, O., Bell, E. B., and Miyan, J. A. (2004). Nerve fibres are required to evoke a contact sensitivity response in mice. *Immunology* 111, 118–125. doi: 10.1111/j.1365-2567.2004.01786.x

Bernard, G. W., and Shih, C. (1990). The osteogenic stimulating effect of neuroactive calcitonin gene-related peptide. *Peptides* 11, 625–632. doi: 10.1016/0196-9781(90)90171-Z

Bhave, G., Zhu, W., Wang, H., Brasier, D. J., Oxford, G. S., and Gereau, R. W. 4th. (2002). cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* 35, 721–731. doi: 10.1016/S0896-6273(02)00802-4

Bienenstock, J., Tomioka, M., Matsuda, H., Stead, R. H., Quinonez, G., Simon, G. T., et al. (1987). The role of mast cells in inflammatory processes: evidence for nerve/mast cell interactions. *Int. Arch. Allergy Appl. Immunol.* 82, 238–243. doi: 10.1159/000234197

Bracci-Laudiero, L., Aloe, L., Buanne, P., Finn, A., Stenfors, C., Vigneti, E., et al. (2002). NGF modulates CGRP synthesis in human B-lymphocytes: a possible anti-inflammatory action of NGF? *J. Neuroimmunol.* 123, 58–65. doi: 10.1016/S0165-5728(01)00475-1

Bracci-Laudiero, L., Aloe, L., Caroleo, M. C., Buanne, P., Costa, N., Starace, G., et al. (2005). Endogenous NGF regulates CGRP expression in human monocytes, and affects HLA-DR and CD86 expression and IL-10 production. *Blood* 106, 3507–3514. doi: 10.1182/blood-2004-10-4055

Brain, S. D., and Grant, A. D. (2004). Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol. Rev.* 84, 903–934. doi: 10.1152/physrev.00037.2003

Brain, W. T., S. D., Tippins, J. R., Morris, H. R., and MacIntyre, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313, 54–56. doi: 10.1038/313054a0

Bressan, E., Peres, K. C., and Tonussi, C. R. (2011). Evidence that LPS-reactive arthritis in rats depends on the glial activity and the fractalkine-TNF-alpha signaling in the spinal cord. *Neuropharmacology* 62, 947–958. doi: 10.1016/j.neuropharm.2011.09.028

Broome, C. S., and Miyan, J. A. (2000). Neuropeptide control of bone marrow neutrophil production. A key axis for neuroimmunomodulation. *Ann. N.Y. Acad. Sci.* 917, 424–434. doi: 10.1111/j.1749-6632.2000.tb05407.x

Broome, C. S., Whetton, A. D., and Miyan, J. A. (2000). Neuropeptide control of bone marrow neutrophil production is mediated by both direct and indirect effects on CFU-GM. *Br. J. Haematol.* 108, 140–150. doi: 10.1046/j.1365-2141.2000.01808.x

Buck, S. H., and Burks, T. F. (1986). The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol. Rev.* 38, 179–226.

Carucci, J. A., Ignatius, R., Wei, Y., Cypess, A. M., Schaer, D. A., Pope, M., et al. (2000). Calcitonin gene-related peptide decreases expression of HLA-DR and CD86 by human dendritic cells and dampens dendritic cell-driven T cell-proliferative responses via the type I calcitonin gene-related peptide receptor. *J. Immunol.* 164, 3494–3499.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816–824. doi: 10.1038/39807

Caviedes-Bucheli, J., Moreno, G. C., Lopez, M. P., Bermeo-Noguera, A. M., Pacheco-Rodriguez, G., Cuellar, A., et al. (2008). Calcitonin gene-related peptide receptor expression in alternatively activated monocytes/macrophages during irreversible pulpitis. *J. Endod.* 34, 945–949. doi: 10.1016/j.joen.2008.05.011

Chuang, H. H., Prescott, E. D., Kong, H., Shields, S., Jordt, S. E., Basbaum, A. I., et al. (2001). Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4, 5)P2-mediated inhibition. *Nature* 411, 957–962. doi: 10.1038/35082088

Constantin, C. E., Mair, N., Sailer, C. A., Andratsch, M., Xu, Z. Z., Blumer, M. J., et al. (2008). Endogenous tumor necrosis factor alpha (TNFalpha) requires TNF receptor type 2 to generate heat hyperalgesia in a mouse cancer model. *J. Neurosci.* 28, 5072–5081. doi: 10.1523/JNEUROSCI.4476-07.2008

Dantzer, R., and Kelley, K. W. (2007). Twenty years of research on cytokine-induced sickness behavior. *Brain Behav. Immun.* 21, 153–160. doi: 10.1016/j.bbi.2006.09.006

De Jonge, F., Van Nassauw, L., Adriaensen, D., Van Meir, F., Miller, H. R., Van Marck, E., et al. (2003). Effect of intestinal inflammation on capsaicin-sensitive afferents in the ileum of Schistosoma mansoni-infected mice. *Histochem. Cell Biol.* 119, 477–484. doi: 10.1007/s00418-003-0532-5

Demirbilek, S., Ersoy, M. O., Karaman, A., Gurbuz, N., Bayraktar, N., and Bayraktar, M. (2004). Small-dose capsaicin reduces systemic inflammatory responses in septic rats. *Anesth. Analg.* 99, 1501–1507. doi: 10.1213/01.ANE.0000132975.02854.65

Denes, A., Boldogkoi, Z., Uhereczky, G., Hornyak, A., Rusvai, M., Palkovits, M., et al. (2005). Central autonomic control of the bone marrow: multisynaptic tract tracing by recombinant pseudorabies virus. *Neuroscience* 134, 947–963. doi: 10.1016/j.neuroscience.2005.03.060

De Winter, B. Y., Bredenoord, A. J., Van Nassauw, L., De Man, J. G., De Schepper, H. U., Timmermans, J. P., et al. (2009). Involvement of afferent neurons in the pathogenesis of endotoxin-induced ileus in mice: role of CGRP and TRPV1 receptors. *Eur. J. Pharmacol.* 615, 177–184. doi: 10.1016/j.ejphar.2009.04.055

Dimitriadou, V., Rouleau, A., Dam Trung Tuong, M., Newlands, G. J., Miller, H. R., Luffau, G., et al. (1994). Functional relationship between mast cells and C-sensitive nerve fibres evidenced by histamine H3-receptor modulation in rat lung and spleen. *Clin. Sci.* 87, 151–163.

Ding, W., Stohl, L. L., Wagner, J. A., and Granstein, R. D. (2008). Calcitonin gene-related peptide biases Langerhans cells toward Th2-type immunity. *J. Immunol.* 181, 6020–6026.

Dong, J., He, Y., Zhang, X., Wang, L., Sun, T., Zhang, M., et al. (2010). Calcitonin gene-related peptide regulates the growth of epidermal stem cells *in vitro*. *Peptides* 31, 1860–1865. doi: 10.1016/j.peptides.2010.07.007

Dunzendorfer, S., Meierhofer, C., and Wiedermann, C. J. (1998). Signaling in neuropeptide-induced migration of human eosinophils. *J. Leukoc. Biol.* 64, 828–834.

Eftekhari, S., Warfvinge, K., Blixt, F. W., and Edvinsson, L. (2013). Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. *J. Pain* 14, 1289–1303. doi: 10.1016/j.jpain.2013.03.010

Engel, M. A., Becker, C., Reeh, P. W., and Neurath, M. F. (2010). Role of sensory neurons in colitis: increasing evidence for a neuroimmune link in the gut. *Inflamm. Bowel Dis.* 17, 1030–1033. doi: 10.1002/ibd.21422

Engel, M. A., Khalil, M., Mueller-Tribbensee, S. M., Becker, C., Neuhuber, W. L., Neurath, M. F., et al. (2012). The proximodistal aggravation of colitis depends on substance P released from TRPV1-expressing sensory neurons. *J. Gastroenterol.* 47, 256–265. doi: 10.1007/s00535-011-0495-6

Engel, M. A., Khalil, M., Siklosi, N., Mueller-Tribbensee, S. M., Neuhuber, W. L., Neurath, M. F., et al. (2011). Opposite effects of substance P and calcitonin gene-related peptide in oxazolone colitis. *Dig. Liver Dis.* 44, 24–29. doi: 10.1016/j.dld.2011.08.030

Feng, G., Xu, X., Wang, Q., Liu, Z., Li, Z., and Liu, G. (2010). The protective effects of calcitonin gene-related peptide on gastric mucosa injury after cerebral ischemia reperfusion in rats. *Regul. Pept.* 160, 121–128. doi: 10.1016/j.regpep.2009.11.008

Fernandez, S., Knopf, M. A., Bjork, S. K., and McGillis, J. P. (2001). Bone marrow-derived macrophages express functional CGRP receptors and respond to CGRP by increasing transcription of c-fos and IL-6 mRNA. *Cell. Immunol.* 209, 140–148. doi: 10.1006/cimm.2001.1795

Fernandez, S., Knopf, M. A., and McGillis, J. P. (2000). Calcitonin-gene related peptide (CGRP) inhibits interleukin-7-induced pre-B cell colony formation. *J. Leukoc. Biol.* 67, 669–676.

Foster, C. A., Mandak, B., Kromer, E., and Rot, A. (1992). Calcitonin gene-related peptide is chemotactic for human T lymphocytes. *Ann. N.Y. Acad. Sci.* 657, 397–404. doi: 10.1111/j.1749-6632.1992.tb22785.x

Fox, F. E., Kubin, M., Cassin, M., Niu, Z., Hosoi, J., Torii, H., et al. (1997). Calcitonin gene-related peptide inhibits proliferation and antigen presentation by human peripheral blood mononuclear cells: effects on B7, interleukin 10, and interleukin 12. *J. Invest. Dermatol.* 108, 43–48. doi: 10.1111/1523-1747.ep12285627

Ganor, Y., Drillet-Dangeard, A. S., Lopalco, L., Tudor, D., Tambussi, G., Delongchamps, N. B., et al. (2013). Calcitonin gene-related peptide inhibits Langerhans cell-mediated HIV-1 transmission. *J. Exp. Med.* 210, 2161–2170. doi: 10.1084/jem.20122349

Gibson, S. J., Polak, J. M., Giaid, A., Hamid, Q. A., Kar, S., Jones, P. M., et al. (1988). Calcitonin gene-related peptide messenger RNA is expressed in sensory neurones of the dorsal root ganglia and also in spinal motoneurones in man and rat. *Neurosci. Lett.* 91, 283–288. doi: 10.1016/0304-3940(88)90694-5

Gim, G. T., Lee, J. H., Park, E., Sung, Y. H., Kim, C. J., Hwang, W. W., et al. (2011). Electroacupuncture attenuates mechanical and warm allodynia through suppression of spinal glial activation in a rat model of neuropathic pain. *Brain Res. Bull.* 86, 403–411. doi: 10.1016/j.brainresbull.2011.09.010

Gomes, R. N., Castro-Faria-Neto, H. C., Bozza, P. T., Soares, M. B., Shoemaker, C. B., David, J. R., et al. (2005) Calcitonin gene-related peptide inhibits local acute inflammation and protects mice against lethal endotoxemia. *Shock* 24, 590–594. doi: 10.1097/01.shk.0000183395.29014.7c

Gunthorpe, M. J., Harries, M. H., Prinjha, R. K., Davis, J. B., and Randall, A. (2000). Voltage- and time-dependent properties of the recombinant rat vanilloid receptor (rVR1). *J. Physiol.* 525(Pt 3), 747–759. doi: 10.1111/j.1469-7793.2000.t01-1-00747.x

Guo, A., Vulchanova, L., Wang, J., Li, X., and Elde, R. (1999). Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur. J. Neurosci.* 11, 946–958. doi: 10.1046/j.1460-9568.1999.00503.x

Han, N., Zhang, D. Y., Wang, T. B., Zhang, P. X., and Jiang, B. G. (2010). Calcitonin gene-related peptide induces proliferation and monocyte chemoattractant protein-1 expression via extracellular signal-regulated kinase activation in rat osteoblasts. *Chin. Med. J. (Engl.)* 123, 1748–1753.

Harzenetter, M. D., Novotny, A. R., Gais, P., Molina, C. A., Altmayr, F., and Holzmann, B. (2007). Negative regulation of TLR responses by the neuropeptide CGRP is mediated by the transcriptional repressor ICER. *J. Immunol.* 179, 607–615.

Hasegawa-Moriyama, M., Kurimoto, T., Nakama, M., Godai, K., Kojima, M., Kuwaki, T., et al. (2013). Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone attenuates inflammatory pain through the induction of heme oxygenase-1 in macrophages. *Pain* 154, 1402–1412. doi: 10.1016/j.pain.2013.04.039

He, Y., Ding, G., Xia, D., Zhu, T., and Fan, S. (2002). [Calcitonin gene-related peptide induces chemokine interleukine-8 synthesis in human monocytes]. *Zhonghua Yi Xue Za Zhi* 82, 131–134.

Helliwell, R. J., McLatchie, L. M., Clarke, M., Winter, J., Bevan, S., and McIntyre, P. (1998). Capsaicin sensitivity is associated with the expression of the vanilloid (capsaicin) receptor (VR1) mRNA in adult rat sensory ganglia. *Neurosci. Lett.* 250, 177–180. doi: 10.1016/S0304-3940(98)00475-3

Holzer, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.* 43, 143–201.

Holzer, P., and Maggi, C. A. (1998). Dissociation of dorsal root ganglion neurons into afferent and efferent-like neurons. *Neuroscience* 86, 389–398.

Holzmann, B. (2013). Antiinflammatory activities of CGRP modulating innate immune responses in health and disease. *Curr. Protein Pept. Sci.* 14, 268–274. doi: 10.2174/13892037113149990046

Horii, M., Orita, S., Nagata, M., Takaso, M., Yamauchi, K., Yamashita, M., et al. (2010). Direct application of the tumor necrosis factor-alpha inhibitor, etanercept, into a punctured intervertebral disc decreases calcitonin gene-related peptide expression in rat dorsal root ganglion neurons. *Spine (Phila Pa 1976)* 36, 80–85. doi: 10.1097/BRS.0b013e3181d4be3c

Hosoi, J., Murphy, G. F., Egan, C. L., Lerner, E. A., Grabbe, S., Asahina, A., et al. (1993). Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. *Nature* 363, 159–163. doi: 10.1038/363159a0

Hua, X. Y., Chen, P., Fox, A., and Myers, R. R. (1996). Involvement of cytokines in lipopolysaccharide-induced facilitation of CGRP release from capsaicin-sensitive nerves in the trachea: studies with interleukin-1beta and tumor necrosis factor-alpha. *J. Neurosci.* 16, 4742–4748.

Huang, W., Rubinstein, J., Prieto, A. R., Thang, L. V., and Wang, D. H. (2009). Transient receptor potential vanilloid gene deletion exacerbates inflammation and atypical cardiac remodeling after myocardial infarction. *Hypertension* 53, 243–250. doi: 10.1161/HYPERTENSIONAHA.108.118349

Isami, K., Haraguchi, K., So, K., Asakura, K., Shirakawa, H., Mori, Y., et al. (2013). Involvement of TRPM2 in peripheral nerve injury-induced infiltration of peripheral immune cells into the spinal cord in mouse neuropathic pain model. *PLoS ONE* 8:e66410. doi: 10.1371/journal.pone.0066410

Jin, X., and Gereau, R. W. 4th. (2006). Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-alpha. *J. Neurosci.* 26, 246–255. doi: 10.1523/JNEUROSCI.3858-05.2006

Jones, R. C. 3rd, Xu, L., and Gebhart, G. F. (2005). The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J. Neurosci.* 25, 10981–10989. doi: 10.1523/JNEUROSCI.0703-05.2005

Jordt, S. E., Tominaga, M., and Julius, D. (2000). Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8134–8139. doi: 10.1073/pnas.100129497

Jusek, G., Reim, D., Tsujikawa, K., and Holzmann, B. (2012). Deficiency of the CGRP receptor component RAMP1 attenuates immunosuppression during the early phase of septic peritonitis. *Immunobiology* 217, 761–767. doi: 10.1016/j.imbio.2012.04.009

Khan, A. A., Diogenes, A., Jeske, N. A., Henry, M. A., Akopian, A., and Hargreaves, K. M. (2008). Tumor necrosis factor alpha enhances the sensitivity of rat trigeminal neurons to capsaicin. *Neuroscience* 155, 503–509. doi: 10.1016/j.neuroscience.2008.05.036

Kulka, M., Sheen, C. H., Tancowny, B. P., Grammer, L. C., and Schleimer, R. P. (2008). Neuropeptides activate human mast cell degranulation and chemokine production. *Immunology* 123, 398–410. doi: 10.1111/j.1365-2567.2007.02705.x

Lee, J., Yamamoto, T., Hayashi, S., Kuramoto, H., and Kadowaki, M. (2013). Enhancement of CGRP sensory afferent innervation in the gut during the development of food allergy in an experimental murine model. *Biochem. Biophys. Res. Commun.* 430, 895–900. doi: 10.1016/j.bbrc.2012.12.058

Lee, S., and Zhang, J. (2012). Heterogeneity of macrophages in injured trigeminal nerves: cytokine/chemokine expressing vs. phagocytic macrophages. *Brain Behav. Immun.* 26, 891–903. doi: 10.1016/j.bbi.2012.03.004

Levite, M. (1998). Neuropeptides, by direct interaction with cells, T, induce cytokine secretion and break the commitment to a distinct T helper phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12544–12549. doi: 10.1073/pnas.95.21.12544

Levite, M. (2000). Nerve-driven immunity. The direct effects of neurotransmitters on T-cell function. *Ann. N.Y. Acad. Sci.* 917, 307–321. doi: 10.1111/j.1749-6632.2000.tb05397.x

Levite, M. (2008). Neurotransmitters activate T-cells and elicit crucial functions via neurotransmitter receptors. *Curr. Opin. Pharmacol.* 8, 460–471. doi: 10.1016/j.coph.2008.05.001

Levite, M., Cahalon, L., Hershkoviz, R., Steinman, L., and Lider, O. (1998). Neuropeptides, via specific receptors, regulate T cell adhesion to fibronectin. *J. Immunol.* 160, 993–1000.

Levite, M., Cahalon, L., Peretz, A., Hershkoviz, R., Sobko, A., Ariel, A., et al. (2000). Extracellular K+ and opening of voltage-gated potassium channels activate T cell integrin function: physical and functional association between Kv1.3 channels and beta 1 integrins. *J. Exp. Med.* 191, 1167–1176. doi: 10.1084/jem.191.7.1167

Levite, M., and Chowers, Y. (2001). Nerve-driven immunity: neuropeptides regulate cytokine secretion of T cells and intestinal epithelial cells in a direct, powerful and contextual manner. *Ann. Oncol.* 12(Suppl. 2), S19–S25. doi: 10.1093/annonc/12.suppl_2.S19

Li, F. J., Zou, Y. Y., Cui, Y., Yin, Y., Guo, G., and Lu, F. G. (2013a). Calcitonin gene-related peptide is a promising marker in ulcerative colitis. *Dig. Dis. Sci.* 58, 686–693. doi: 10.1007/s10620-012-2406-y

Li, P., Sun, H. J., Han, Y., Wang, J. J., Zhang, F., Tang, C. S., et al. (2013b). Intermedin enhances sympathetic outflow via receptor-mediated cAMP/PKA signaling pathway in nucleus tractus solitarii of rats. *Peptides* 47, 1–6. doi: 10.1016/j.peptides.2013.05.002

Li, Y., Guan, C., Zhou, Y., Sun, G., Shi, M., Wu, J., et al. (2010). Effect of calcitonin gene-related peptide on the expression of triggering receptor expressed on myeloid cells-1 in lipopolysaccharide-induced macrophages. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 35, 100–106. doi: 10.3969/j.issn.1672-7347.2010.02.002

Linscheid, P., Seboek, D., Schaer, D. J., Zulewski, H., Keller, U., and Muller, B. (2004). Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes. *Crit. Care Med.* 32, 1715–1721. doi: 10.1097/01.CCM.0000134404.63292.71

Luo, X. J., Li, N. S., Zhang, Y. S., Liu, B., Yang, Z. C., Li, Y. J., et al. (2011). Vanillyl nonanoate protects rat gastric mucosa from ethanol-induced injury through a mechanism involving calcitonin gene-related peptide. *Eur. J. Pharmacol.* 666, 211–217. doi: 10.1016/j.ejphar.2011.05.032

Ma, W., Dumont, Y., Vercauteren, F., and Quirion, R. (2010). Lipopolysaccharide induces calcitonin gene-related peptide in the RAW264.7 macrophage cell line. *Immunology* 130, 399–409. doi: 10.1111/j.1365-2567.2009.03239.x

Mach, D. B., Rogers, S. D., Sabino, M. C., Luger, N. M., Schwei, M. J., Pomonis, J. D., et al. (2002). Origins of skeletal pain: sensory and sympathetic innervation of the mouse femur. *Neuroscience* 113, 155–166. doi: 10.1016/S0306-4522(02)00165-3

Massa, F., Sibaev, A., Marsicano, G., Blaudzun, H., Storr, M., and Lutz, B. (2006). Vanilloid receptor (TRPV1)-deficient mice show increased susceptibility to dinitrobenzene sulfonic acid induced colitis. *Mol. Med. J. (Berl.)* 84, 142–146. doi: 10.1007/s00109-005-0016-2

Matsuda, R., Kezuka, T., Nishiyama, C., Usui, Y., Matsunaga, Y., Okunuki, Y., et al. (2012). Suppression of murine experimental autoimmune optic neuritis by mature dendritic cells transfected with calcitonin gene-related Peptide gene. *Invest. Ophthalmol. Vis. Sci.* 53, 5475–5485. doi: 10.1167/iovs.12-9935

McGillis, J. P., Humphreys, S., Rangnekar, V., and Ciallella, J. (1993). Modulation of B lymphocyte differentiation by calcitonin gene-related peptide (CGRP). II. Inhibition of LPS-induced kappa light chain expression by CGRP. *Cell Immunol.* 150, 405–416. doi: 10.1006/cimm.1993.1208

McGillis, J. P., Rangnekar, V., and Ciallella, J. R. (1995). A role for calcitonin gene related peptide (CGRP) in the regulation of early B lymphocyte differentiation. *Can. J. Physiol. Pharmacol.* 73, 1057–1064. doi: 10.1139/y95-150

Mikami, N., Matsushita, H., Kato, T., Kawasaki, R., Sawazaki, T., Kishimoto, T., et al. (2011). Calcitonin gene-related peptide is an important regulator of cutaneous immunity: effect on dendritic cell and T cell functions. *J. Immunol.* 186, 6886–6893. doi: 10.4049/jimmunol.1100028

Mikami, N., Miyagi, Y., Sueda, K., Takatsuji, M., Fukada, S., Yamamoto, H., et al. (2013). Calcitonin gene-related peptide and cyclic adenosine 5'-monophosphate/protein kinase A pathway promote IL-9 production in Th9 differentiation process. *J. Immunol.* 190, 4046–4055. doi: 10.4049/jimmunol.1203102

Mikami, N., Fukada, S., Yamamoto, H., and Tsujikawa, K. (2012b). [The regulatory mechanisms of calcitonin gene-related peptide (CGRP) in skin inflammation]. *Yakugaku Zasshi* 132, 1211–1215. doi: 10.1248/yakushi.12-00232-1

Mikami, N., Watanabe, K., Hashimoto, N., Miyagi, Y., Sueda, K., Fukada, S., et al. (2012a). Calcitonin gene-related peptide enhances experimental autoimmune encephalomyelitis by promoting Th17-cell functions. *Int. Immunol.* 24, 681–691. doi: 10.1093/intimm/dxs075

Monneret, G., Arpin, M., Venet, F., Maghni, K., Debard, A. L., Pachot, A., et al. (2003). Calcitonin gene related peptide and N-procalcitonin modulate CD11b upregulation in lipopolysaccharide activated monocytes and neutrophils. *Intensive Care Med.* 29, 923–928.

Morrison, J. F., Dhanasekaran, S., and Howarth, F. C. (2009). Neuropeptides in the rat corpus cavernosum and seminal vesicle: effects of age and two types of diabetes. *Auton. Neurosci.* 146, 76–80. doi: 10.1016/j.autneu.2008.11.016

Mourad, F. H., Barada, K. A., Khoury, C., Hamdi, T., Saade, N. E., and Nassar, C. F. (2009). Amino acids in the rat intestinal lumen regulate their own absorption from a distant intestinal site. *Am. J. Physiol. Gastrointest. Liver Physiol.* 297, G292–G298. doi: 10.1152/ajpgi.00100.2009

Mullins, M. W., Ciallella, J., Rangnekar, V., and McGillis, J. P. (1993). Characterization of a calcitonin gene-related peptide (CGRP) receptor on mouse bone marrow cells. *Regul. Pept.* 49, 65–72. doi: 10.1016/0167-0115(93)90385-L

Olah, Z., Karai, L., and Iadarola, M. J. (2001). Anandamide activates vanilloid receptor 1 (VR1) at acidic pH in dorsal root ganglia neurons and cells ectopically expressing VR1. *J. Biol. Chem.* 276, 31163–31170. doi: 10.1074/jbc.M101607200

Olesen, J. (2011a). Calcitonin gene-related peptide (CGRP) in migraine. *Cephalalgia* 31, 510. doi: 10.1177/0333102410388438

Olesen, J. (2011b). CGRP in migraine. *Cephalalgia* 31, 638. doi: 10.1177/0333102410395790

Origuchi, T., Iwamoto, N., Kawashiri, S. Y., Fujikawa, K., Aramaki, T., Tamai, M., et al. (2010). Reduction in serum levels of substance P in patients with rheumatoid arthritis by etanercept, a tumor necrosis factor inhibitor. *Mod. Rheumatol.* 21, 244–250. doi: 10.1007/s10165-010-0384-5

Ostrowski, S. M., Belkadi, A., Loyd, C. M., Diaconu, D., and Ward, N. L. (2011). Cutaneous denervation of psoriasiform mouse skin improves acanthosis and inflammation in a sensory neuropeptide-dependent manner. *J. Invest. Dermatol.* 131, 1530–1538. doi: 10.1038/jid.2011.60

Peng, J., and Li, Y. J. (2009). The vanilloid receptor TRPV1, role in cardiovascular and gastrointestinal protection. *Eur. J. Pharmacol.* 627, 1–7. doi: 10.1016/j.ejphar.2009.10.053

Petitto, J. M., Huang, Z., and McCarthy, D. B. (1994). Molecular cloning of NPY-Y1 receptor cDNA from rat splenic lymphocytes: evidence of low levels of mRNA expression and [125I]NPY binding sites. *J. Neuroimmunol.* 54, 81–86. doi: 10.1016/0165-5728(94)90234-8

Ren, J. Y., Song, J. X., Lu, M. Y., and Chen, H. (2011). Cardioprotection by ischemic postconditioning is lost in isolated perfused heart from diabetic rats: involvement of transient receptor potential vanilloid 1, calcitonin gene-related peptide and substance P. *Regul. Pept.* 169, 49–57. doi: 10.1016/j.regpep.2011.04.004

Rochlitzer, S., Veres, T. Z., Kuhne, K., Prenzler, F., Pilzner, C., Knothe, S., et al. (2011). The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function. *Clin. Exp. Allergy* 41, 1609–1621. doi: 10.1111/j.1365-2222.2011.03822.x

Roggenkamp, D., Kopnick, S., Stab, F., Wenck, H., Schmelz, M., and Neufang, G. (2013). Epidermal nerve fibers modulate keratinocyte growth via neuropeptide signaling in an innervated skin model. *J. Invest. Dermatol.* 133, 1620–1628. doi: 10.1038/jid.2012.464

Rosas-Ballina, M., Olofsson, P. S., Ochani, M., Valdes-Ferrer, S. I., Levine, Y. A., Reardon, C., et al. (2011). Acetylcholine-synthesizing T Cells relay neural signals in a vagus nerve circuit. *Science* 334, 98–101. doi: 10.1126/science.1209985

Saito, O., Svensson, C. I., Buczynski, M. W., Wegner, K., Hua, X. Y., Codeluppi, S., et al. (2010). Spinal glial TLR4-mediated nociception and production of prostaglandin E(2) and TNF. *Br. J. Pharmacol.* 160, 1754–1764. doi: 10.1111/j.1476-5381.2010.00811.x

Santambrogio, L., Lipartiti, M., Bruni, A.,and Dal Toso, R. (1993). Dopamine receptors on human T- and B-lymphocytes. *J. Neuroimmunol.* 45, 113–119. doi: 10.1016/0165-5728(93)90170-4

Schafer, M. K., Eiden, L. E., and Weihe, E. (1998). Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. II. The peripheral nervous system. *Neuroscience* 84, 361–376. doi: 10.1016/S0306-4522(97)80196-0

Schafers, M., Sommer, C., Geis, C., Hagenacker, T., Vandenabeele, P., and Sorkin, L. S. (2008). Selective stimulation of either tumor necrosis factor receptor differentially induces pain behavior *in vivo* and ectopic activity in sensory neurons *in vitro*. *Neuroscience* 157, 414–423. doi: 10.1016/j.neuroscience.2008.08.067

Schlomer, J. J., Storey, B. B., Ciornei, R. T., and McGillis, J. P. (2007). Calcitonin gene-related peptide inhibits early B cell development *in vivo*. *J. Leukoc. Biol.* 81, 802–808. doi: 10.1189/jlb.0306229

Shepherd, A. J., Beresford, L. J., Bell, E. B., and Miyan, J. A. (2005a). Mobilisation of specific T cells from lymph nodes in contact sensitivity requires substance P. *J. Neuroimmunol.* 164, 115–123. doi: 10.1016/j.jneuroim.2005.04.008

Shepherd, A. J., Downing, J. E., and Miyan, J. A. (2005b). Without nerves, immunology remains incomplete -*in vivo* veritas. *Immunology* 116, 145–163. doi: 10.1111/j.1365-2567.2005.02223.x

Smith, A. S., and Smid, S. D. (2005). Impaired capsaicin and neurokinin-evoked colonic motility in inflammatory bowel disease. *J. Gastroenterol. Hepatol.* 20, 697–704. doi: 10.1111/j.1440-1746.2005.03759.x

Sommer, C., Schmidt, C., and George, A. (1998). Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1. *Exp. Neurol.* 151, 138–142. doi: 10.1006/exnr.1998.6797

Sorkin, L. S., and Doom, C. M. (2000). Epineurial application of TNF elicits an acute mechanical hyperalgesia in the awake rat. *J. Peripher. Nerv. Syst.* 5, 96–100. doi: 10.1046/j.1529-8027.2000.00012.x

Springer, J., Geppetti, P., Fischer, A., and Groneberg, D. A. (2003). Calcitonin gene-related peptide as inflammatory mediator. *Pulm. Pharmacol. Ther.* 16, 121–130. doi: 10.1016/S1094-5539(03)00049-X

Szallasi, A. (1995). Autoradiographic visualization and pharmacological characterization of vanilloid (capsaicin) receptors in several species, including man. *Acta Physiol. Scand. Suppl.* 629, 1–68.

Szallasi, A., and Blumberg, P. M. (1999). Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.* 51, 159–212.

Szolcsanyi, J., and Bartho, L. (2001). Capsaicin-sensitive afferents and their role in gastroprotection: an update. *J. Physiol. Paris* 95, 181–188. doi: 10.1016/S0928-4257(01)00023-7

Talme, T., Liu, Z., and Sundqvist, K. G. (2008). The neuropeptide calcitonin gene-related peptide (CGRP) stimulates T cell migration into collagen matrices. *J. Neuroimmunol.* 196, 60–66. doi: 10.1016/j.jneuroim.2008.02.007

Taves, S., Berta, T., Chen, G., and Ji, R. R. (2013). Microglia and spinal cord synaptic plasticity in persistent pain. *Neural Plast.* 2013, 753656. doi: 10.1155/2013/753656

Teodorczyk-Injeyan, J. A., Triano, J. J., McGregor, M., Woodhouse, L., and Injeyan, H. S. (2011). Elevated production of inflammatory mediators including nociceptive chemokines in patients with neck pain: a cross-sectional evaluation. *J. Manipulative Physiol. Ther.* 34, 498–505. doi: 10.1016/j.jmpt.2011.08.010

Tokoyoda, K., Tsujikawa, K., Matsushita, H., Ono, Y., Hayashi, T., Harada, Y., et al. (2004). Up-regulation of IL-4 production by the activated cAMP/cAMP-dependent protein kinase (protein kinase A) pathway in CD3/CD28-stimulated naive T cells. *Int. Immunol.* 16, 643–653. doi: 10.1093/intimm/dxh072

Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., et al. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 21, 531–543. doi: 10.1016/S0896-6273(00)80564-4

Toth, B. I., Benko, S., Szollosi, A. G., Kovacs, L., Rajnavolgyi, E., and Biro, T. (2009). Transient receptor potential vanilloid-1 signaling inhibits differentiation and activation of human dendritic cells. *FEBS Lett.* 583, 1619–1624. doi: 10.1016/j.febslet.2009.04.031

Tsuji, F., Murai, M., Oki, K., Seki, I., Ueda, K., Inoue, H., et al. (2009). Transient receptor potential vanilloid 1 agonists as candidates for anti-inflammatory and immunomodulatory agents. *Eur. J. Pharmacol.* 627, 332–339. doi: 10.1016/j.ejphar.2009.10.044

Tsujikawa, K., Yayama, K., Hayashi, T., Matsushita, H., Yamaguchi, T., Shigeno, T., et al. (2007). Hypertension and dysregulated proinflammatory cytokine production in receptor activity-modifying protein 1-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16702–16707. doi: 10.1073/pnas.0705974104

Umeda, Y., Takamiya, M., Yoshizaki, H., and Arisawa, M. (1988). Inhibition of mitogen-stimulated T lymphocyte proliferation by calcitonin gene-related peptide. *Biochem. Biophys. Res. Commun.* 154, 227–235. doi: 10.1016/0006-291X(88)90674-2

Van Nassauw, L., Adriaensen, D., and Timmermans, J. P. (2007). The bidirectional communication between neurons and mast cells within the gastrointestinal tract. *Auton. Neurosci.* 133, 91–103. doi: 10.1016/j.autneu.2006.10.003

Voedisch, S., Rochlitzer, S., Veres, T. Z., Spies, E., and Braun, A. (2012). Neuropeptides control the dynamic behavior of airway mucosal dendritic cells. *PLoS ONE* 7:e45951. doi: 10.1371/journal.pone.0045951

Vyklicky, L., Knotkova-Urbancova, H., Vitaskova, Z., Vlachova, V., Kress, M., and Reeh, P. W. (1998). Inflammatory mediators at acidic pH activate capsaicin receptors in cultured sensory neurons from newborn rats. *J. Neurophysiol.* 79, 670–676.

Wang, F., Millet, I., Bottomly, K., and Vignery, A. (1992). Calcitonin gene-related peptide inhibits interleukin 2 production by murine T lymphocytes. *J. Biol. Chem.* 267, 21052–21057.

Wang, X., Xing, L., Xing, Y., Tang, Y., and Han, C. (1999). Identification and characterization of immunoreactive calcitonin gene-related peptide from lymphocytes of the rat. *J. Neuroimmunol.* 94, 95–102. doi: 10.1016/S0165-5728(98)00230-6

Wong, C. W., O, W. S., and Tang, F. (2013). Intermedin in rat uterus: changes in gene expression and peptide levels across the estrous cycle and its effects on uterine contraction. *Reprod. Biol. Endocrinol.* 11, 13. doi: 10.1186/1477-7827-11-13

Yiangou, Y., Facer, P., Ford, A., Brady, C., Wiseman, O., Fowler, C. J., et al. (2001). Capsaicin receptor VR1 and ATP-gated ion channel P2X3 in human urinary bladder. *BJU Int.* 87, 774–779. doi: 10.1046/j.1464-410x.2001.02190.x

Zhang, L., Berta, T., Xu, Z. Z., Liu, T., Park, J. Y., and Ji, R. R. (2010a). TNF-alpha contributes to spinal cord synaptic plasticity and inflammatory pain: distinct role of TNF receptor subtypes 1 and 2. *Pain* 152, 419–427. doi: 10.1016/j.pain.2010.11.014

Zhang, X. C., Kainz, V., Burstein, R., and Levy, D. (2010b). Tumor necrosis factor-alpha induces sensitization of meningeal nociceptors mediated via local COX and p38 MAP kinase actions. *Pain* 152, 140–149. doi: 10.1016/j.pain.2010.10.002

Zhou, Y., Zhang, M., Sun, G. Y., Liu, Y. P., Ran, W. Z., Peng, L., et al. (2013). Calcitonin gene-related peptide promotes the wound healing of human bronchial epithelial cells via PKC and MAPK pathways. *Regul. Pept.* 184, 22–29. doi: 10.1016/j.regpep.2013.03.020

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 October 2013; accepted: 27 January 2014; published online: 14 February 2014.

*Citation: Assas BM, Pennock JI and Miyan JA (2014) Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. Front. Neurosci. **8**:23. doi: 10.3389/fnins.2014.00023*

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in
NEUROSCIENCE

REVIEW ARTICLE
published: 06 February 2014
doi: 10.3389/fnins.2014.00012

Kynurenines in CNS disease: regulation by inflammatory cytokines

Brian M. Campbell, Erik Charych, Anna W. Lee and Thomas Möller*

Neuroinflammation Disease Biology Unit, Lundbeck Research USA, Paramus, NJ, USA

Edited by:
Adam Denes, University of Manchester, UK

Reviewed by:
Robert Schwarcz, Maryland Psychiatric Research Center, USA
Robert Dantzer, MD Anderson Cancer Center, USA

****Correspondence:***
Thomas Möller, Neuroinflammation Disease Biology Unit, Lundbeck Research USA, 215 College Rd., Paramus, NJ 07652, USA
e-mail: tmoe@lundbeck.com

The kynurenine pathway (KP) metabolizes the essential amino acid tryptophan and generates a number of neuroactive metabolites collectively called the kynurenines. Segregated into at least two distinct branches, often termed the "neurotoxic" and "neuroprotective" arms of the KP, they are regulated by the two enzymes kynurenine 3-monooxygenase and kynurenine aminotransferase, respectively. Interestingly, several enzymes in the pathway are under tight control of inflammatory mediators. Recent years have seen a tremendous increase in our understanding of neuroinflammation in CNS disease. This review will focus on the regulation of the KP by inflammatory mediators as it pertains to neurodegenerative and psychiatric disorders.

Keywords: kynurenine, neuroinflammation, microglia, astrocytes, CNS disease, IDO, KMO, KAT

THE KYNURENINE PATHWAY

The metabolic fate of tryptophan (TRP), an essential amino acid, is conversion into a variety of neuroactive substances including the well-known neurotransmitters serotonin and melatonin, as well as a range of kynurenine metabolites such as kynurenic acid (KYNA), 3-hydroxykynurenine (3-HK), and quinolinic acid (QUIN). Enzymes involved in the metabolism of tryptophan along the kynurenine pathway (KP) are located thoughout the body and brain. Though the highest levels are found in the liver and kidney, all of the primary enzymes are also found within the brain. Kynurenine metabolism occurs in all cells within the brain, though various branches of the pathway appear segregated into specific cell types (Heyes et al., 1997; Amori et al., 2009). The first and rate-limiting enzyme into the KP is indole-2,3-dioxygenase (IDO), and to a lesser extent in the brain tryptophan-2,3-dioxygenase (TDO), which convert tryptophan to N-formylkynurenine (Shimizu et al., 1978; Takikawa et al., 1988) (for a schematic of the pathway see **Figure 1**). N-formylkynurenine is then metabolized to l-kynurenine (L-KYN) by kynurenine formamidase at which point the pathway bifurcates into at least two distinct branches regulated by kynurenine monooxygenase (KMO) and kynurenine aminotransferases (KATs I-IV). The majority of kynurenine metabolism within the brain takes place in glia. KMO, kynureninase (KYNU), and 3-hydroxyanthranillic acid oxidase (3-HAO) regulate production of a host of metabolites in microglia leading to formation of anthranillic acid (AA), 3-hydroxy anthranillic acid (3-HAA), 3-HK, and QUIN. QUIN is, an excitatory (excitotoxic) agent at NMDA-type glutamate receptors and synergizes with 3-HK to produce oxidative stress. Alternatively, L-KYN may be metabolized in astrocytes by KATs, with KAT II being the predominant brain subtype in humans and rats (Guidetti et al., 2007a). KATs convert L-KYN to KYNA, an inhibitor of glutamate neurotransmission and possibly an antagonist at nicotinic α_7 receptors. The endogenous function of kynurenine-derived neuroactive metabolites still requires further research since many have multiple receptor targets. In addition to NMDA and nicotinic a7 receptors, KYNA for example is reported to interact with GPR35 (Wang et al., 2006) and arylhydrocarbon receptors (Dinatale et al., 2010). A third possible pathway regulated by both KMO and KATs is the xanthurenic acid (XA) branch. Little is known about the endogenous function of XA, though recent literature indicates that it is a Group II metabotropic glutamate receptor agonist (Copeland et al., 2013) indicating that it could also regulate glutamate neurotransmission by impacting presynaptic release.

In recent years the regulation of kynurenine metabolism has been intensely evaluated as it relates to CNS disorders (Haroon et al., 2012; Schwarcz et al., 2012). Often termed the "neurotoxic" and "neuroprotective" branches of the KP, or alternatively the "excitatory" and "inhibitory" branches, KMO and KATs regulate the balance of QUIN:KYNA production which is important in both neurodegenerative and psychiatric disorders. Many kynurenine-derived metabolites poorly cross the blood brain barrier implying that CNS concentrations of kynurenine metabolites are largely regulated by local enzyme activity (Gal and Sherman, 1978). However, kynurenine itself is actively transported into the brain by the large neutral amino acid transporter (Fukui et al., 1991). Under normal physiological conditions much of the kynurenine which is converted to QUIN and KYNA in the brain is derived from peripheral sources (Kita et al., 2002). Following systemic inflammation, where IDO expression is greatly increased (Moreau et al., 2008; Macchiarulo et al., 2009), nearly all kynurenine in the CNS comes from the periphery. However, in contrast to this, direct induction of neuroinflammation causes >98% of the kynurenine available for metabolism in the brain to be derived from local production (Kita et al., 2002). The current review will evaluate this interplay between proinflammatory mediators and mechanisms by which they regulate the KP. It will then conclude with a review of the role of neuroinflammation-mediated kynurenine dysregulation in a range of neurodegenerative and psychiatric disorders.

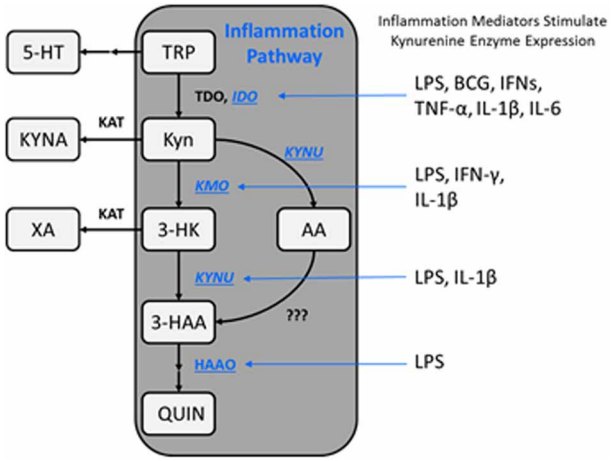


FIGURE 1 | Schematic representation of the kynurenine metabolic pathway. The kynurenine pathway is commonly segregated into two distinct branches that are regulated by KATs and KMO, as well as the availability of l-kynurenine within the brain. Additionally, kynurenine metabolism is regulated by a variety of proinflammatory mediators which impact enzyme expression levels, thereby altering substrate availability and metabolite formation favoring the KMO branch of the pathway under immune-related pathological conditions. TRP, tryptophan; 5-HT, serotonin; Kyn, kynurenine; KYNA, kynurenine acid; 3-HK, 3-hydroxykynurenine; AA, anthranilic acid; XA, xanthurenic acid; 3-HAA, 3-hydroxyanthranilic acid; QUIN, quinolinic acid; IDO, indoleamine-2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine 3- monooxygenase; KYNU, kynureninase; HAAO, 3-hydroxyanthranilic acid oxidase; LPS, lipopolysaccharide; BCG, bacillus Calmette-Guerin; IFNs, interferons; TNF, tumor necrosis factor; IL, interleukin.

CYTOKINE-MEDIATED REGULATION OF KYNURENINE METABOLISM

IDO and TDO, which initiate the catabolism of tryptophan toward kynurenine, are generally thought to be regulated by different mechanisms. TDO is induced by corticosteroids and glucagon, while IDO is induced by proinflammatory cytokines during an immune response (Lestage et al., 2002). There is some evidence that TDO can also be induced by immune activation but this is suggested to be mediated indirectly by increased glucocorticoid receptor activation (Walker et al., 2013). While there is some evidence that other enzymes within the excitatory branch of the KP can also be induced by proinflammatory cytokines, the regulation of IDO, particularly by interferon (IFN)-γ, has been examined most extensively. In general, the body of work investigating the regulation of KP enzymes by inflammatory cytokine signaling is largely composed of expression studies and therefore must be interpreted with caution, since changes in mRNA or even protein expression are not necessarily indicative of functional changes in enzyme activity.

EFFECTS OF PROINFLAMMATORY MEDIATORS ON INDOLEAMINE 2,3-DIOXYGENASE (IDO)

IDO is expressed in various immune cells throughout the body, including dendritic cells, monocytes, macrophages, and, importantly in microglia, the resident CNS macrophage-like cell population (Mandi and Vecsei, 2012). IDO is preferentially induced by interferons and by IFN-inducers such as lipopolysaccharide (LPS) and viruses (Musso et al., 1994). IFN-γ, a type II interferon, is the predominant cytokine implicated in the induction of IDO, as has been shown in several myeloid cell types including dendritic cells, monocytes, immortalized murine macrophages, and microglia (Alberati-Giani et al., 1996; Fujigaki et al., 2006; Jung et al., 2007; O'connor et al., 2009a). In human macrophages, IDO expression and QUIN production can also be induced by the type 1 interferons, IFN-α and IFN-β, although to a lesser degree than with IFN-γ (Jansen and Reinhard, 1999; Guillemin et al., 2001). In the bacille Calmette-Guérin (BCG) mouse model of chronic inflammation, IDO induction closely parallels increased IFN-γ and tumor necrosis factor alpha (TNF-α) expression (Moreau et al., 2005, 2008). BCG-induced upregulation of IDO mRNA is completely inhibited in IFN-γR$^{-/-}$ mice, along with an associated lack of IDO activity, demonstrating that IFN-γ receptor function is necessary for BCG-induced IDO activation (O'connor et al., 2009a).

Although IFN-γ is regarded as the primary inducer of IDO, there is some evidence that IDO expression can be induced independently of IFN-γ. Systemic LPS administration induces IDO expression in rat cortex and hippocampus accompanied by a robust increase in central TNF-α and interleukin (IL)-6 expression, but only modestly elevated IFN-γ (Connor et al., 2008). In the same paper, similar findings were reported in mixed glia cultures prepared from neonatal rat cortex suggesting that IFN-γ may not be necessary for LPS-induced IDO expression (Connor et al., 2008). Consistent with this finding, *in vitro* data with THP-1 cells, a human monocytic cell line, indicate that LPS-induced IDO activation can be mediated by an IFN-γ-independent mechanism involving synergistic effects of IL-1β, TNF-α, and IL-6 (Fujigaki et al., 2006). In human hippocampal progenitor cells, treatment with IL-1β greatly upregulated the transcript for IDO, but not TDO (Zunszain et al., 2012). The increase in IDO transcript was associated with a decrease in tryptophan and increase in kynurenine in the supernatant suggesting that IL-1β increased levels of functional IDO enzyme (Zunszain et al., 2012).

Studies examining the effects of anti-inflammatory cytokines on IDO expression are limited and often conflicting, likely due to differences in the cellular models used and experimental conditions applied. For example, the prototypical anti-inflammatory cytokine IL-10 dose-dependently decreased LPS-mediated IDO protein expression in mouse bone marrow-derived dendritic cells (BMDCs), whereas IL-10 enhanced IFN-γ-mediated IDO protein expression in these cells (Jung et al., 2009; Yanagawa et al., 2009). This discrepancy may point to the possibility that distinct mechanisms of IDO induction may be differentially regulated by anti-inflammatory cytokines such as IL-10, though whether this occurs in the CNS has not been determined. Interestingly, however, IL-10 suppressed IFN-γ-mediated IDO mRNA induction in GT1-7 cells, a transformed mouse hypothalamic neuronal cell line, contrary to that reported for mouse BMDCs treated with IFN-γ (Tu et al., 2005). In addition to the prototypical anti-inflammatory cytokine IL-10, studies with human monocytes and fibroblasts have demonstrated that IL-4 inhibits the induction of IDO mRNA and IDO activity by IFN-γ. In contrast, a study using the EOC13.31 mouse microglia cell line found that IL-4

enhanced, rather than suppressed, IFN-γ-induced IDO mRNA expression, which was abolished by the addition of IL-4 antiserum (Yadav et al., 2007). The potentiating effect of IL-4 on IFN-γ-induced IDO expression was also observed at the level of protein expression and enzymatic activity in these cells (Yadav et al., 2007). Furthermore, IL-4, as well as IL-13 which signals through the same receptor subunit, potentiated IFN-γ-mediated IDO expression in primary mouse microglia cultures (Yadav et al., 2007). These findings collectively suggest that microglia respond differently to anti-inflammatory cytokines compared to peripheral myeloid cells. Interestingly, central administration of IL-4 exacerbates the depressive-like behavioral effect of peripheral LPS, which is IDO-dependent, when both IL-4 and LPS are delivered simultaneously, but suppresses the depressive effect when administered 12 h before LPS, highlighting the complex relationship between IL-4 and IDO in the CNS (Bluthe et al., 2002).

IFN-γ-dependent mechanisms of IDO induction

The 5′-flanking region of the human gene encoding IDO (*INDO*) contains several regulatory elements including some that are essential for IFN-γ-mediated gene transcription. One of two identified IFN-γ-activated sites (GAS) and two interferon-sensitive response elements (ISREs), the latter highly homologous to that associated with IFN-α-inducible genes, are required for full induction of IDO by IFN-γ (Dai and Gupta, 1990; Hassanain et al., 1993; Chon et al., 1995, 1996; Konan and Taylor, 1996). As shown in **Figure 2**, canonical IFN-γ-mediated signal transduction leads to (1) tyrosine phosphorylation of STAT-1, triggering its dimerization and translocation to the nucleus where it binds the GAS sequence in the 5′-flanking region of *INDO*, and (2) NF-κB- and STAT-1-dependent synthesis of IFN-γ-regulated factor (IRF)-1, which binds to one or both of the ISREs found in the *INDO* 5′-flanking region (Darnell et al., 1994; Chon et al., 1995, 1996; Konan and Taylor, 1996). Thus, cooperative STAT-1 and IRF-1 binding to GAS and ISRE sequences, respectively, within the *INDO* 5′-flanking region are necessary for full IFN-γ-mediated induction of IDO transcription.

Synergistic mechanisms of IFN-γ-mediated IDO Induction

The regulatory mechanisms for IFN-γ-mediated IDO induction can be potentiated by other proinflammatory cytokines such as TNF-α and IL-1β, and toll-like receptor (TLR) agonists such as LPS, resulting in synergistic enhancement of IDO expression (Hu et al., 1995; Hissong and Carlin, 1997; Babcock and Carlin, 2000; Currier et al., 2000; Robinson et al., 2003). IL-1β and TNF-α can enhance the expression of IFN-γ receptor in an NF-κB-dependent manner, thereby lowering the threshold for IDO induction by IFN-γ (Krakauer and Oppenheim, 1993; Shirey et al., 2006). Moreover, together with IFN-γ, TNF-α synergistically induces IDO expression by increasing both STAT-1 activation and NF-κB-dependent IRF-1 expression (Krakauer and Oppenheim, 1993; Ohmori et al., 1997; Robinson et al., 2003, 2006; Shirey et al.,

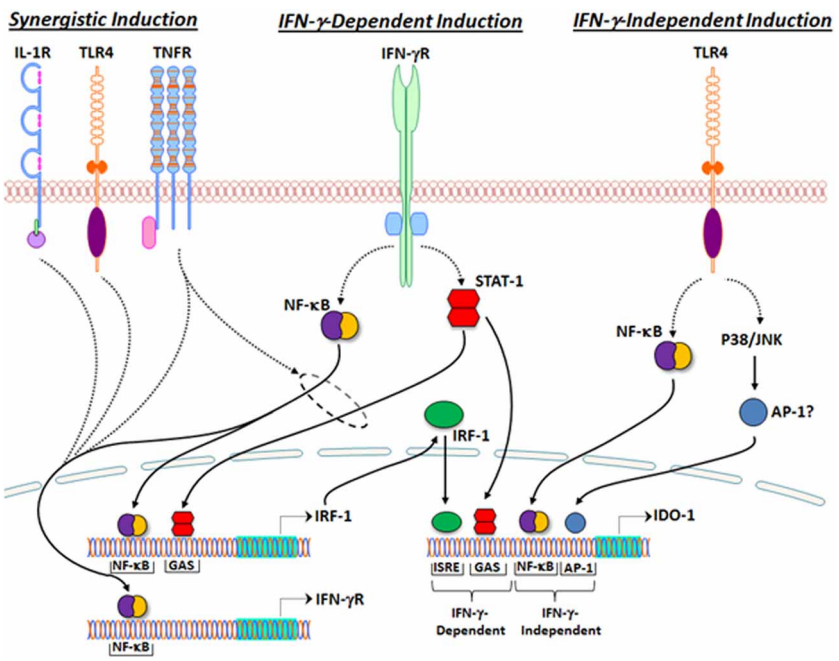


FIGURE 2 | Regulation of IDO1 transcription by inflammatory signaling. IFN-γ-dependent IDO1 induction (**middle**). Canonical IFN-γ receptor signal transduction leads to (1) NF-κB- and STAT-1-dependent transcription of IRF-1, and (2) IRF-1- and STAT-1-dependent transcription of IDO1. Synergistic IDO1 induction (**Left**). IL-1β, LPS, and TNF-α enhance transcription of IFN-γ receptor in an NF-κB-dependent manner. TNF-α has been shown to synergistically enhance IFN-γ-dependent IDO1 transcription by promoting NF-κB- and STAT-1-dependent IRF-1 transcription (within dashed circle). IFN-γ-Independent IDO induction (**Right**). TLR4 stimulation by LPS leads to transcription of IDO1 by a mechanism that requires NF-κB and either p38 or JNK, but not IFN-γ. The 5′-flanking region of *INDO*, the gene encoding IDO1, contains two IFN-γ-activated sites (GAS) and two interferon-sensitive response elements (ISREs). One of the two GAS sequences and both ISRE sequences are required for IFN-γ-mediated IDO1 induction. The 5′ flanking region of *INDO* also contains at least one NF-κB binding site and several AP-1 binding sites, which may be required for IFN-γ-independent mechanisms of IDO1 transcription.

2006). Given the requirement for both STAT-1 and IRF-1 binding to ISRE and GAS sequences, respectively, presumably other signaling mechanisms that increase both STAT-1 phosphorylation and NF-κB transactivation may also synergize with IFN-γ to enhance IDO induction, though these mechanisms have not yet been directly tested. Interestingly, the synergistic induction of IDO by IFN-γ and TNF-α occurs in primary murine microglia and, furthermore, *in vivo* studies suggest that this synergy participates in the IDO-mediated generation of depressive-like behavior in mice inoculated with BCG (O'connor et al., 2009a), a model of inflammation-related depression (Moreau et al., 2008).

IFN-γ-independent mechanisms of IDO induction

Studies using primary murine microglia demonstrated that LPS stimulates IDO transcription in an IFN-γ-independent manner, since IDO mRNA levels were enhanced but IFN-γ mRNA was undetectable following LPS stimulation in these cells (Connor et al., 2008; Wang et al., 2010). Furthermore, these studies showed that LPS-stimulated IDO induction was attenuated by an inhibitor of c-Jun-N-terminal kinase (JNK) (Wang et al., 2010). Similar studies using THP-1 cells, demonstrated that LPS-stimulated L-KYN production was not accompanied by STAT-1 or IRF-1 binding activities, but was attenuated by p38 and NF-κB inhibitors (Fujigaki et al., 2001, 2006). Collectively, these data suggest that LPS-stimulated IDO induction in monocyte/macrophage-like cells occurs in an IFN-γ-independent manner and involves NF-κB and stress-activated mitogen-activated protein (MAP) kinases such as p38 and JNK (Fujigaki et al., 2001, 2006, 2012; Wang et al., 2010). The downstream mechanisms leading from p38 or JNK activation to IDO induction in response to LPS stimulation have not been elucidated. However, the AP-1 transcription factors are conventional substrates of both p38 and JNK MAPKs and are important regulators of inflammation-related gene transcription (Huang et al., 2009; Wang et al., 2010). Supporting this possibility, a reanalysis of the 5′-flanking region of *INDO* has identified both NF-κB and several AP-1 recognition sequences, consistent with the participation of both NF-κB and stress-activated MAPK activity in LPS-stimulated IDO induction (Fujigaki et al., 2006; Wang et al., 2010).

In addition to TLR4 agonists such as LPS, the TLR3 agonist polyinosinic:polycytidylic acid (polyI:C) can induce IDO transcription in cultured human astrocytes in a manner dependent on IFN-β but not IFN-γ signaling, and requiring both NF-κB and IRF-3 (Suh et al., 2007). Though these signaling components have been shown to participate in astrocyte IDO induction, it is not yet clear whether the corresponding mechanism can be generalized to cell types other than astrocytes since the effect of TLR3 activation on IDO induction has not been demonstrated elsewhere.

Aryl hydrocarbon receptor-dependent IDO induction

Experiments using murine BMDCs have demonstrated that the TLR4 and TLR9 agonists LPS and CpG, respectively, induce expression of the aryl hydrocarbon receptor (AhR). The AhR is a ligand-gated transcription factor belonging to the basic helix-loop-helix Per-Arnt-Sim (PAS) family, widely known as the dioxin receptor (Vogel et al., 2008; Nguyen et al., 2010; Vondracek et al., 2011). Interestingly, these experiments suggested that LPS- or CpG-stimulated IDO induction was entirely dependent on the co-induction of AhR in these cells, since BMDCs derived from $AhR^{-/-}$ mice lost the ability to induce IDO expression in response to treatment with either LPS or CpG (Nguyen et al., 2010). Furthermore, dioxin, a potent agonist of the AhR, can also induce IDO expression in these cells, suggesting that AhR activation may positively regulate IDO transcription in response to TLR4 or TLR9 stimulation (Nguyen et al., 2010). Intriguingly, AhR-mediated IDO induction may act as a positive feedback mechanism further activating AhR since L-KYN and its metabolite KYNA are themselves potent AhR agonists (Dinatale et al., 2010; Opitz et al., 2011). The AhR exerts its effects on gene transcription through nuclear translocation and direct binding to dioxin response elements (DREs) in the promoter region of target genes. Curiously these elements have not been identified in the promotor region of *INDO*. Thus, it is not clear whether AhR can regulate IDO transcription directly or indirectly in these cells.

EFFECTS OF PROINFLAMMATORY MEDIATORS ON KYNURENINE-3-MONOOXYGENASE (KMO)

Aside from IDO, the regulation of other kynurenine enzymes by proinflammatory cytokines has not been studied extensively. However, studies are emerging indicating that, similar to IDO, enzymes within the KMO branch of the pathway may also be induced by proinflammatory stimuli. KMO expression is increased in rat brain after systemic LPS administration (Connor et al., 2008; Molteni et al., 2013). In a study that examined the effects of IFN-γ treatment on immortalized murine macrophage (MT2) and microglia (N11) cells, KMO was induced in both cells types, KYNU was induced only in MT2 macrophages, and 3-HAO was not effected (Alberati-Giani et al., 1996). Finally, in human hippocampal progenitor cells, IL-1β treatment upregulated the level of transcripts for KMO and KYNU, enzymes in the KMO branch of the pathway (Zunszain et al., 2012).

EFFECTS OF PROINFLAMMATORY MEDIATORS ON KYNURENINE AMINOTRANSFERASES (KATs)

While the expression of IDO and kynurenine enzymes in the excitatory branch of the KP are either elevated or not changed by proinflammatory stimuli, KAT expression is either unaffected or decreased. Systemic LPS administration had no effect on KAT II expression in rat brain (Connor et al., 2008; Molteni et al., 2013). In MT2 macrophage and N11 microglia cells, KAT appeared to be constitutively expressed, but there was no effect of IFN-γ treatment on KAT activity (Alberati-Giani et al., 1996). However, since in the CNS KATs are mainly expressed in astrocytes, further studies on the effects of proinflammatory stimuli on KAT expression and activity using relevant cell types are required. In human hippocampal progenitor cells, KAT I and KAT III, but not KAT II mRNA, were downregulated after IL-1β treatment (Zunszain et al., 2012).

DYSREGULATION OF THE KYNURENINE PATHWAY IN CNS DISEASES

In recent years dysregulation of kynurenine metabolism has been described in a wide range of CNS-related disorders. Several

studies have demonstrated that altered cytokine levels and associated dysregulation of kynurenine metabolism plays as important role in the pathophysiology of neurodegenerative diseases and psychiatric disorders. Upregulation of kynurenines are observed in the serum, CSF and/or brain in neurodegenerative diseases (e.g., AD, PD, and HD), autoimmune diseases (e.g., MS), epilepsy, psychiatric diseases (e.g., MDD, schizophrenia, and ADHD) and infectious diseases (e.g., HIV-associated neurocognitive disorder). It is generally predicted that diseases where microglia are activated favor production of 3-HK and QUIN, whereas suppression of this branch or astrocyte activation may favor KYNA synthesis. The following sections will review the role of the kynurenine system and its regulation by cytokines in the pathophysiology of diseases, and discuss potential therapeutic interventions targeting the KP.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurological disorder characterized by impaired memory, cognitive decline, and dementia. Currently there is still only a limited understanding of AD etiology, particularly in late onset AD. AD pathology hallmarks are the presence of β-amyloid (Aβ) plaques, neurofibrillary tangles, and gliosis. Multiple hypotheses exist regarding factors that contribute to the development and progression of AD including substantial evidence for neuroinflammatory processes. In fact, microglia activation states correlate with disease progression and levels of dementia (Arends et al., 2000; Cagnin et al., 2006). Analysis of serum samples and post-mortem brain tissue from AD patients demonstrate an imbalance in pro- and anti-inflammatory cytokines, as well as irregular tryptophan metabolism through activation of microglia and astrocytes.

(Neuro)inflammatory state in AD

Among the neurochemical changes in AD, IFN-γ, TNF-α, IL-1β, IL-2, and IL-8 are elevated along with lower levels of tryptophan and increased kynurenine levels in serum samples from AD patients (Widner et al., 1999; Alsadany et al., 2013; Niranjan, 2013). Similar changes are found in post-mortem brain tissue along with IL-6 also increased (Huell et al., 1995). Within the brains of AD patients, activated microglia and astrocytes are found in proximity to neuritic plaques. Treatment of human microglia and monocytes with $A\beta_{1-42}$ induces IDO expression (Guillemin et al., 2003) and primes the cells for synergistic induction of the KP by IFN-γ (Yamada et al., 2009). In astrocytes Aβ only modestly stimulated IL-6 and IL-8 secretion, but primed the cells to markedly respond to IL-1β with a 3–8 fold increase in IL-6 and IL-8 release (Gitter et al., 1995). Similarly, exposure of microglia cultures from AD patients to $A\beta_{1-42}$ induced TNF-α, pro-IL-1β, IL-6, and IL-8 (Lue et al., 2001). Thus, Aβ appears to alter the state of microglia to a more proinflammatory phenotype that may contribute to neuronal dysfunction and ultimately cell death through release of cytokines and free radical generating agents including NO and QUIN. In AD brains IDO was associated with senile plaques and was localized with neurofibrillary tangles (Bonda et al., 2010). Additionally, IDO and QUIN immunoreactivity were increased in microglia, astrocytes, and neurons within the hippocampus of AD patients (Guillemin et al., 2005) which is of particular interest since QUIN may cause tau hyperphosphorylation in human cortical neurons (Rahman et al., 2009).

Inflammation and kynurenine metabolism in animal models of AD

Studies in preclinical models support the hypothesis that induction of kynurenine metabolism by Aβ and/or cytokines may contribute to neural pathology in AD. Elevated $A\beta_{1-40}$ and $A\beta_{1-42}$ found in transgenic AD mice were associated with increased TNF-α, IL-6, and IL-1β (Patel et al., 2005). In Tg2576 mice, basal induction of IDO in activated microglia associated with Aβ plaques appears to be low, though robustly increased following stimulation with LPS suggesting that the cells are in a "primed" state ready to respond to immune challenges in a more durable way than WT controls (Akimoto et al., 2007). QUIN was strongly increased in the hippocampus, but not cerebellum, in a progressive and age dependent manner in triple transgenic mice (3×Tg: PS1M146V, APPSwe, and tauP301L) in line with data showing increased TDO and IDO-1 immunoreactivity in AD hippocampal tissue (Wu et al., 2013). Interestingly, modest but significant increases in TDO mRNA and protein, along with robust increases in 3-HAO were also found in the cerebellum of these mice, however, 3-HAA levels were unfortunately not reported. Furthermore, TDO was also colocalized with QUIN, neurofibrillary tangles, and amyloid deposits in 3×Tg mice. Recent studies using available pharmacological agents in AAPtg mice showed that chronic inhibition of KMO reduced synaptic loss as measured by synaptophysin, prevented spatial memory deficits in the Morris water maze, and reversed anxiogenic-like responses in the elevated plus maze (Zwilling et al., 2011). Together these data support the hypothesis that Aβ- and cytokine-mediated induction of kynurenine metabolism is an important link in the pathophysiological development of AD.

Induction of kynurenine metabolism, particularly along the KMO/QUIN branch of the pathway, appears likely in AD. The mechanism by which this happens, and the functional consequences, is still under investigation. To date, much of the data available is correlative indicating that Aβ is able to induce production of cytokines and kynurenine metabolizing enzymes which may both contribute to synaptic dysfunction and neuronal loss. Whether Aβ in AD brains independently induces cytokine production and kynurenine metabolism, activates cytokine release which in turn stimulates kynurenine production, or "primes" glia such that they are able to more robustly respond to cytokine signaling is currently not well-understood. Whichever the case, evidence is emerging that excess production of proinflammatory cytokines and QUIN by glia contribute to the progression, and perhaps etiology, of AD.

Preclinical evidence supports the use of IDO, TDO, KMO, and/or 3-HAO inhibitors to counteract the effects of neuroinflammation in AD. However, the contribution of peripheral vs. central inflammatory processes in any putative kynurenine-related AD pathology is not yet clear, and few of the tools available to test these hypotheses are able to directly target the brain. Association of IDO and TDO with plaques and tangles suggests that brain permeable drugs are needed, though peripheral inhibition of KMO was sufficient to produce a therapeutic

effect in Tg2576 mice. Since the source of kynurenine feeding into the QUIN branch differs substantially under basal, systemic inflammation, or neuroinflammation conditions, understanding the contribution of central vs. peripheral (including endothelial cells at the blood brain barrier) kynurenine production will be important to help define an appropriate intervention strategy in AD.

PARKINSON'S DISEASE

Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder characterized by loss of dopaminergic neurons in the midbrain and presence of protein inclusions called Lewy bodies (Zinger et al., 2011). The detailed pathogenesis of PD is not known, but several mechanisms have been proposed including mitochondrial dysfunction, neurotoxicity from excessive glutamatergic activity, and reactive oxygen species. Neuroinflammation, as measured by the presence of activated microglia in PD brain, as well as excessive production of cytokines and dysregulation of the KP have been suggested to be involved in these complex pathogenic events.

(Neuro)inflammatory state in PD

Many studies support the presence of widespread microglia activation in PD. In two such studies, MHC class II expression, a widely used marker of microglial activation, was assessed in PD post-mortem brain (McGeer et al., 1988; Imamura et al., 2003). The number of MHC class II-positive microglia was higher in the substantia nigra and putamen as well as in the hippocampus, transentorhinal cortex, cingulate cortex, and temporal cortex of PD brains, and frequently in association with α-synuclein-positive Lewy neurites and monoaminergic neurites (McGeer et al., 1988; Imamura et al., 2003). These activated microglia were also positive for TNF-α and IL-6 in the putamen of PD brain (Imamura et al., 2003). *In vivo* imaging of microglia activation with [^{11}C](R)-PK11195 PET in PD revealed widespread activation in brain regions including the pons, basal ganglia, and frontal and temporal cortex (Gerhard et al., 2006). Levels of several cytokines including TNF-α, IL-1β, IL-2, IL-4, IL-6, and transforming growth factor (TGF)-alpha have been shown to be elevated in the CSF and striatum of PD brain (Mogi et al., 1994a,b; Nagatsu et al., 2000). Some of these cytokines are known inducers or amplifiers of the KP and might contribute to the dysregulation of KPs in PD.

Dysregulation of kynurenine metabolites in PD

Changes in kynurenine metabolism have been reported in post-mortem PD brain and mouse models of PD. In mouse models of PD, mice injected with the dopaminergic neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine have diminished KAT-I immunoreactivity in the pars compacta of the substania nigra (Knyihar-Csillik et al., 2004, 2006). Treatment with the metabolite of MPTP, 1-methyl-4-phenylpyridinium ion (MPP^+), dose-dependently decreased KAT-II activity and KYNA concentration in rat cerebral cortical slices (Luchowski et al., 2002). Similar to the KYNA changes observed in rodent models of PD, KYNA levels were reported to be decreased in PD post-mortem brain (Ogawa et al., 1992). In contrast, levels of 3-HK were elevated in the frontal cortex, putamen, and pars compacta of the substantia nigra in PD brain (Ogawa et al., 1992). In terms of IDO activity, as measured by the K/T ratio, there were increases in both serum and CSF of PD patients compared to controls (Widner et al., 2002). Taken together, these studies suggest that there is an imbalance between the two main branches of the KP in PD, favoring kynurenine metabolism toward the KMO branch of the pathway.

Potential therapeutic intervention by modulation of kynurenine pathway

Numerous studies have been conducted demonstrating that modulation of the KP by enhancing KYNA and/or decreasing 3-HK and QUIN is a potential therapeutic strategy for PD. In an *in vitro* PD model, pretreatment with KYNA attenuated MPP^+-induced neurotoxicity in human neuroblastoma cell lines (Lee Do et al., 2008). In rats, KYNA injection into the brain prevented QUIN-induced reduction in striatal tyrosine hydroxylase activity, suggesting that KYNA can protect dopaminergic neurons against QUIN or NMDA-mediated excitotoxicity (Miranda et al., 1997). Since KYNA does not cross the blood brain barrier, investigators in one study attempted to increase KYNA levels in the brain with systemic injections of the substrate for KYNA, L-KYN, in combination with probenecid, an inhibitor of organic acid transport (Silva-Adaya et al., 2011). They reported that pretreatment with L-KYN and probenecid had a protective effect on 6-OHDA-induced locomotor asymmetry, striatal reactive gliosis and neurodegeneration, and changes in dopamine levels (Silva-Adaya et al., 2011). In another study, four synthetic kynurenine analogs were demonstrated to have beneficial effects in the MPTP model in mice (Acuna-Castroviejo et al., 2011). Finally, in MPTP-treated primates, intracerebral injections of KYNA alleviated symptoms of akinesia, tremor, and rigidity in MPTP-treated animals (Graham et al., 1990). Thus, KYNA or its analogs have been demonstrated to have neuroprotective effects in PD.

The effect of decreasing metabolites in the KMO branch of the KP and thereby increasing KYNA in the brain has been tested pharmacologically by several investigators. When nicotinylalanine, an inhibitor of both KMO and kynureninase, was administered intraventricularly in combination with systemic L-KYN and probenecid, there was an elevation in brain KYNA levels and protective effects against QUIN-induced toxicity in rats (Miranda et al., 1997). The effect of KMO inhibition has been tested in a variety of animal models of PD with the non-brain penetrant KMO inhibitor Ro 61-6048. Ro 61-6048 given either systemically or intrastriatally, reduced the severity of dystonia in dt^{sz} mutant hamsters, which are used as an animal model of paroxysmal dystonia with striatal dysfunctions (Richter and Hamann, 2003; Hamann et al., 2008). In non-human primates, chronic Ro 61-8048 administration reduced the development of levodopa-induced dyskinesia in MPTP-treated animals (Samadi et al., 2005; Gregoire et al., 2008; Ouattara et al., 2009; Tamim et al., 2010). Taken together, these studies indicate that drug development targeting the KP for PD may be a promising opportunity.

HUNTINGTON'S DISEASE

Huntington's disease (HD) is an adult-onset neurodegenerative disorder caused by expansion of a CAG repeat in the gene

encoding the huntingtin (Htt) protein. Several mechanisms that are not mutually exclusive have been suggested to play a role in the pathogenesis of HD. These mechanisms include neuroinflammation, transcriptional dysregulation, excitotoxicity, and mitochondrial dysfunction. Damage of mutant Htt expressing neurons is suggested to lead to microglia activation, which includes secretion of cytokines as well as increased IDO transcription and generation of neuroactive kynurenine metabolites (Schwarcz and Pellicciari, 2002). Indeed, increased levels of metabolites have been reported in human post-mortem brain as well as in various animal models of HD as discussed below.

(Neuro)inflammatory state in HD

Several lines of evidence suggest both peripheral and central immune systems are activated in HD. Activation of the peripheral immune system is indicated by elevation in several plasma cytokines in HD patients including IL-6, IL-8, IL-4, IL-10, and TNF-α (Bjorkqvist et al., 2008). Interestingly, plasma levels of IL-6 were elevated on average 16 years prior to the predicted clinical onset of the disease in HD gene carriers, which is the earliest plasma abnormality identified in HD (Bjorkqvist et al., 2008). In a study that examined blood levels of kynurenine metabolites at different stages of HD, levels of IL-2 were found to be correlated with the K/T ratio, disease severity, and number of CAG repeats (Forrest et al., 2010).

Activation of the immune system in the CNS is evidenced by elevations in IL-6 and IL-8 in the CSF and increased expression of these inflammatory transcripts, as well as by increased TNF-α in post-mortem HD brain (Bjorkqvist et al., 2008; Silvestroni et al., 2009). There is ample evidence that microglia, the main mediators of neuroinflammation, contribute to the progressive neurodegeneration observed in HD (Möller, 2010). Interestingly they are also the main producers of 3-HK and QUIN in the CNS. Given the presence of IDO and KMO inducing enzymes and the data showing increased KP metabolism in HD and HD model brains, it is tempting to speculate that an increased flux through the microglial KMO metabolic pathway might be responsible for these observations.

Dysregulation of kynurenine metabolites in HD

Studies examining post-mortem HD brain found elevations in the levels of 3-HK and QUIN (Pearson and Reynolds, 1992; Guidetti et al., 2000, 2004). The activity of 3-HAO, the biosynthetic enzyme in the metabolism of 3-HAA, was increased in HD brains compared to controls, suggesting that the HD brain has the ability to produce elevated levels of QUIN (Schwarcz et al., 1988). On the other hand, levels of KYNA and the activity of its two biosynthetic enzymes (KAT I and KAT II) were reported to be reduced in HD brain and CSF compared to controls (Beal et al., 1990, 1992; Jauch et al., 1995) suggesting a dysregulation of the KP in the brain away from KYNA and toward QUIN.

R6/2 mice, a well-established model of HD, also have elevated 3-HK in the brain and have increased activity of the biosynthetic enzyme of 3-HK, KMO, which may account for the high levels (Guidetti et al., 2006; Sathyasaikumar et al., 2010). YAC128 transgenic mice, which have the full-length mutant Htt protein and show a similar degree of striatal neurodegeneration observed in early stage HD, have elevated 3-HK and QUIN in the brain (Guidetti et al., 2000, 2006). Intriguingly, QUIN injections into the striatum is commonly used as an experimental model of HD and produces cellular, neurochemical and behavioral changes resembling those observed in human HD (Beal et al., 1991; Huang et al., 1995).

Dysregulation of the KP, as measured by the K/T ratio, a marker of IDO activity, has been reported in the periphery as well (Stoy et al., 2005; Forrest et al., 2010). One study examined levels of kynurenine metabolites in the blood of patients at different stages of HD as well as the number of CAG repeats and found blood levels of K/T ratio were correlated with disease severity and the number of CAG trinucleotide repeats in HD patients (Forrest et al., 2010). In the same study, blood levels of anthranilic acid were correlated with the proinflammatory cytokine IL-23 (Forrest et al., 2010). Taken together, these studies suggest a role of dysregulation of the KP in HD which may be related to the degree of clinical disease severity.

Potential therapeutic intervention by modulation of kynurenine pathway in Huntington's disease

Studies in yeast, flies, and mice, have shown that blockade of the KMO branch of the KP, thus increasing KYNA in the brain, may protect against neurodegeneration. Genetic deletion of KMO in yeast cells engineered to over express mutated huntingtin protein reduced polyglutamine-mediated toxicity as well as generation of the neuroactive kynurenine metabolites 3HK and QUIN (Giorgini et al., 2005). Furthermore, when a high throughput screen was conducted on the yeast model an analog of the KMO inhibitor 3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]benzenesulfonamide (Ro61-8048) was identified that potently suppressed huntingtin-mediated toxicity (Giorgini et al., 2005). In transgenic *Drosophila melanogaster* flies that express mutant Htt protein, genetic or pharmacological blockade of KMO reduced neuronal cell loss (Campesan et al., 2011). In the R6/2 genetic mouse model of HD, peripheral blockade of KMO increased KYNA in the brain, reduced loss of synapses and microglia activation, and improved survival (Zwilling et al., 2011). In N171-82Q mice, another transgenic animal model of HD, a KYNA analog, N-(2-N,N-dimethylaminoethyl)-4-oxo-1H-quinoline2-carboxamide hydrochloride, was found to be neuroprotective as it prolonged survival, ameliorated hypolocomotion, prevented weight loss, and completely prevented the atrophy of the striatal neurons (Zadori et al., 2011). These investigations suggest that KMO inhibition and/or KYNA enhancement may be neuroprotective in HD and lend support for the KP as potential drug targets.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic, demyelinating autoimmune disease of the CNS characterized by the presence of peripheral immune cells within sites of active demyelination (Carson, 2002). Based on human studies combined with experimental autoimmune encephalomyelitis (EAE), a widely-employed rodent model of MS, it is generally hypothesized that invading T-cells reactive to myelin-specific antigens are the principle effectors in MS pathogenesis (Lassmann and Ransohoff, 2004; Petermann and Korn,

2011; Fuvesi et al., 2012). Thus, a primary aim of MS research has been to define effector T-cell subpopulations relevant to disease pathogenesis and the mechanisms regulating their differentiation. As will be discussed below, accumulating evidence suggests that KP activity (1) is altered in a manner that is temporally related to the clinical course and treatment of the disease, (2) may play a role in autoimmunity by regulating T-cell differentiation, and (3) may influence the cross-talk of auto-reactive T-cells with resident microglia and infiltrating macrophages and dendritic cells.

Human studies implicating kynurenine pathway modulation in multiple sclerosis

Evidence for altered KP metabolism in MS first appeared in 1979 with the finding that TRP levels were significantly reduced in both plasma and CSF samples from MS patients compared with those of control subjects [Monaco et al., 1979; but see Ott et al. (1993)]. More recent studies have shown that, relative to control subjects, the downstream KP metabolite KYNA is significantly decreased in CSF of MS patients during remission, but elevated in the CSF and plasma of MS patients undergoing acute clinical exacerbation (Rejdak et al., 2002, 2007; Hartai et al., 2005). While this putative relationship between KYNA production and clinical phase has not been confirmed by single longitudinal studies, it has nevertheless collectively led to the speculation that such changes in KYNA levels during disease progression and remission reflect a compensatory protective mechanism against excitatory neurotoxicity. This hypothesis derives from the view that, as a putative NMDAR antagonist, the primary function of central KYNA is neuroprotective. However, this has not been directly tested in rodent models such as EAE as of yet. Nevertheless, these findings highlight the possibility that KP metabolism is related to the occurrence of MS and, importantly, to clinical phases of the disease.

A small number of studies have also related changes in KP metabolism to therapeutic intervention in MS patients. Therapeutically relevant concentrations of IFN-β, a standard fist-line immunomodulatory treatment for MS, leads to induction of IDO mRNA and a significant increase in the production of QUIN by human monocyte-derived macrophages (Guillemin et al., 2001). In MS patients, treatment with IFN-β leads to significant acute elevations in plasma or serum L-KYN levels and K/T ratio compared to baseline measurements, consistent with the induction of IDO in response to IFN-β (Amirkhani et al., 2005; Durastanti et al., 2011). Given the hypothesized role of KP metabolism in the mechanism underlying the depressive side-effects associated with IFN-α-based immunotherapy (Bonaccorso et al., 2002a), KP activation may be similarly involved in the depressive side-effects often reported for MS patients undergoing IFN-β treatment (Goeb et al., 2006). However, the precise relationship between IFN-β treatment and depressive symptoms in MS has not yet been definitively established, hindered in part by the partial overlap of MS symptoms with those of depression (Goeb et al., 2006). Moreover, in studies that have examined the occurrence of depressive symptoms in the context of IFN-β treatment for MS, the role that changes in KP metabolism may play has not been explored.

It has also been postulated that IFN-β-mediated IDO induction might contribute to the limited efficacy of IFN-β treatment in improving MS symptomatology (Vecsei et al., 2013). However, this idea is based on the *in vitro* finding that IFN-β leads to the production of QUIN in human monocyte-derived macrophages (Guillemin et al., 2001), incorporating the notion that QUIN is excitotoxic in the CNS (Vecsei et al., 2013). To date, though, there is no evidence that therapeutic IFN-β treatment in MS leads to central QUIN elevation as a result of IDO induction. In fact, it is not yet clear in which cell-type(s) the IFN-β-mediated IDO induction occurs in MS patients, nor which downstream KP branch is primarily affected.

Mechanistic insights into the role of the kynurenine pathway in multiple sclerosis: lessons from the EAE model

Since resident microglial activation and macrophage infiltration into the CNS are common features of both MS and EAE, initial interest in the role of KP metabolism in the pathogenesis of EAE arose from findings that cultured human macrophages can produce QUIN at neurotoxic levels in response to acute treatment with IFN-γ (Heyes et al., 1992; Chiarugi et al., 2001a). Indeed, in rats immunized with myelin basic protein (MBP) to induce EAE, the spinal cord concentration of QUIN is elevated compared to control rats with a time-course that closely follows the development of acute neurological symptoms, returning to control levels during remission (Flanagan et al., 1995). This presumably results from induction of IDO, but also of KMO, since anti-KMO immunoreactivity, KMO enzyme activity, as well as tissue levels of 3-HK and QUIN are enhanced in the spinal cords of EAE compared to control rats (Chiarugi et al., 2001b). Interestingly, treatment of EAE rats with the selective KMO inhibitor Ro 61-8048 significantly attenuates spinal cord 3-HK and QUIN and enhances L-KYN and KYNA, but does not alter the symptom severity in these animals (Chiarugi et al., 2001b). This observation seems to argue against a role of QUIN-mediated neurotoxicity and KYNA-mediated neuroprotection in acute clinical exacerbation and remission, respectively, in EAE and potentially MS. It does not, however, preclude a cumulative role for 3-HK and QUIN in the chronic neurodegeneration associated with secondary progressive MS.

Contrary to a contributing role in acute pathogenesis, mounting evidence from numerous EAE studies implicates IDO and specific KP metabolites in limiting autoimmunity and promoting immune tolerance, which might, in part, account for the periodic remissions observed in MS and EAE. In mice immunized with MBP or proteolipid protein 139–151 ($PLP_{139-151}$), brain and spinal cord K/T ratio, as well as IDO mRNA and protein expression within brain and spinal cord microglia/macrophages, progressively increases with the development of EAE compared to control mice (Sakurai et al., 2002; Kwidzinski et al., 2005). However, an opposing reduction in brain and spinal cord IFN-γ mRNA during the development of EAE (Sakurai et al., 2002) suggests that IDO activity may negatively regulate the survival of IFN-γ-producing T helper type 1 (Th1) cells, thought to be a primary pathogenic T-cell subset in both MS and EAE. Consistent with this hypothesis, inhibition of IDO enzymatic activity with 1-methyl- tryptophan (1-MT) was associated with earlier relapse phase onset, significantly greater maximum clinical score, and more extensive myelitis in spinal cords of EAE mice (Sakurai et al.,

2002). Similarly, EAE mice treated with 1-MT exhibit greater clinical scores during both relapse and remission phases, compared to EAE mice treated with vehicle control (Kwidzinski et al., 2005). Eliminating the possibility of off-target effects by 1-MT on exacerbation of EAE (Agaugue et al., 2006), IDO$^{-/-}$ EAE mice exhibit more severe clinical scores compared to wildtype EAE mice, beginning approximately 2 weeks post-immunization with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ (Yan et al., 2010). Moreover, IDO$^{-/-}$ mice exhibit enhanced Th1/Th17-like cytokine profiles, two major T-cell phenotypes implicated in EAE-related autoimmunity, accompanying the exacerbation of clinical symptoms in these mutants (Yan et al., 2010). Thus, a model of IDO-mediated negative feedback in EAE is emerging. IFN-γ produced by accumulating autoreactive T-cells leads to IDO induction within local antigen presenting cells (APCs), such as microglia or infiltrating macrophages and dendritic cells. This, in turn, limits the survival of pathogenic T-cell phenotypes (i.e., Th1 and Th17) and/or promotes the expansion of immunoregulatory T-cell phenotypes (i.e., Th2 and regulatory T-cells [Treg]).

A firmly established mechanism by which IDO induction may limit the survival of pathogenic T-cells is by directly reducing local availability of TRP, since it has been shown that IDO induction in macrophages and dendritic cells suppresses T-cell proliferation by local TRP catabolism (Munn et al., 1998, 1999; Mellor et al., 2003). Thus, IFN-γ-mediated IDO induction within local APCs may provide an immunosuppressive environment to control self-tolerance during inflammation. In addition to the local reduction of TRP, KP metabolites 3-hydroxykynurenic acid (3-HKA, a.k.a. xanthurenic acid), N-(3,4-dimethoxycinnamoyl) anthranilic acid (3,4-DAA), the synthetic orally active 3-HAA derivative, and 3-HAA directly suppress the proliferation of myelin-specific T-cells, specifically inhibiting Th1 and/or Th17-like phenotypes, and improving EAE clinical symptoms (Platten et al., 2005; Yan et al., 2010). At least for Th17 suppression, 3-HAA enhances the expression of TGF-β in dendritic cells (DCs), stimulating the differentiation of Tregs from naïve T-cells (Tnaïve) (Yan et al., 2010). Thus, KP metabolism may suppress autoimmunity in EAE not only through local TRP depletion, but also through the influence of KP metabolites on DC-mediated T-cell differentiation.

Though the cellular sources of the 3-HAA that act on DCs to influence T-cell differentiation is not clear, it is likely that one source of 3-HAA, or other relevant KP metabolites, may be DCs themselves since bone marrow stem cell (BMSC)-induced down-regulation of EAE correlates with IDO induction in CD11c^{+} DCs (Matysiak et al., 2008). Intriguingly, IDO induction in BMDCs and, as a consequence, Treg differentiation in BMDC/Tnaïve cocultures, requires AhR, the ligands of which include L-KYN, KYNA, and possibly other KP metabolites (Nguyen et al., 2010). In AhR$^{-/-}$ BMDCs cocultured with Tnaïve cells, the inability of these BMDCs to induce Treg differentiation is rescued by addition of L-KYN, though it cannot be excluded that the effect of L-KYN on Treg generation is not a direct effect on Tnaïve cells (Nguyen et al., 2010) since L-KYN can also lead to AhR-dependent Treg differentiation in isolated Tnaïve cells (Mezrich et al., 2010). This may nevertheless have implications for EAE since AhR can bidirectionally drive T-cell differentiation either toward Treg or Th17 phenotypes, ameliorating or worsening EAE, respectively, depending on the specific AhR ligand (Quintana et al., 2008, 2010; Veldhoen et al., 2008). Though the effects of specific KP metabolites on AhR-mediated T-cell differentiation has not been tested directly in EAE, it is still tempting to speculate that metabolites such as 3-HAA and L-KYN might ameliorate EAE through AhR-mediated Treg differentiation, either indirectly by stimulating DC TGF-β release, or directly within Tnaïve cells.

Potential therapeutic intervention by modulation of kynurenine pathway in multiple sclerosis

The emerging model of KP metabolism in the underlying biology of EAE and potentially MS suggests that IDO activity, enhanced by IFN-γ released from pathogenic T-cells, may in turn serve to limit their survival and/or facilitate the expansion of immunoregulatory T-cell phenotypes during inflammation. This is postulated to occur directly through the impact of TRP catabolism on Th1/Th17 cell survival and/or by the influence of downstream KP metabolites on T-cell differentiation toward immunoregulatory phenotypes. Given the compelling positive link between IDO activity and major depressive symptoms, highlighted by clinical studies examining the depressive side-effects of IFN-α-based immunotherapy (Bonaccorso et al., 2002a), a more favorable therapeutic entry-point for MS might be based on the hypothesis that selected downstream KP metabolites serve to limit autoimmunity by influencing T-cell differentiation toward regulatory phenotypes. This hypothesis has been tested in EAE with the synthetic 3-HAA derivative N-(3,4-dimethoxycinnamoyl) anthranilic acid (3,4-DAA), also known as Tranilast, currently approved in the U.S. for the treatment of allergic rhinitis, atopic dermatitis, and certain forms of asthma (Platten et al., 2005; Chen and Guillemin, 2009; Yan et al., 2010). However, Tranilast is also proposed to inhibit histamine release by mast cells, suppress TGF-β release, and inhibit angiogenesis (Chen and Guillemin, 2009). Thus, deeper investigation into the mechanism underlying the influence of KP metabolites on T-cell differentiation may further define novel and more selective therapeutic strategies for treating autoimmune diseases such as MS in this context. To the best of our knowledge, Tranilast is currently being developed by Nuon Therapeutics, Inc. (San Mateo, CA) for the treatment of autoimmune diseases including MS, though it has not entered clinical testing.

EPILEPSY

Research efforts to investigate the function and therapeutic potential of CNS KP metabolism was originally rooted in speculation about the pro- and anti-convulsant properties of endogenous QUIN and KYNA, respectively, in the etiology of human epilepsies (Perkins and Stone, 1985; Stone and Connick, 1985; Schwarcz et al., 1987). However, in over 25 years since these ideas surfaced, surprisingly little evidence has accumulated to date, neither clinical nor experimental, to solidify alterations in KP metabolism as a major etiological factor in human epilepsy. Furthermore, the therapeutic potential of experimental KP modulators such as Ro 61-8048 and various KYNA analogs in epilepsy treatment has not been fully explored (Vecsei et al., 2013). Given this, it is not surprising that even less is known about the regulation of KP

metabolism by inflammatory mediators in this context. Though outside the scope of this review, it is becoming increasingly apparent that proinflammatory cytokine signaling plays a prominent role in the mechanisms underlying neuronal hyperexcitability and neurodegeneration in epilepsy, and has been extensively reviewed elsewhere (Devinsky et al., 2013; Vezzani et al., 2013a,b). Several studies suggest that the impact of epilepsy-related neuroinflammation on KP metabolism as a disease mechanism warrants deeper investigation.

A recent study analyzed serum K/T ratios in 271 classified epilepsy patients with 309 control subjects (Liimatainen et al., 2011). Results were consistent with elevated IDO activity in patients with idiopathic generalized epilepsy (Liimatainen et al., 2011). The central KP metabolites produced downstream of IDO activation in these patients may likely be biased toward the KMO branch since microglial activation is evident in surgical resections from several forms of epilepsy (Vezzani et al., 2013a). Furthermore, in mice inoculated with hamster neurotrophic measles virus, increases in microglial activation and brain levels of 3-HK and QUIN precede the onset of behavioral seizures in this model (Lehrmann et al., 2008). Consistent with the induction of microglial IDO and KMO by proinflammatory cytokine signaling in a mouse model of temporal lobe epilepsy, hippocampal elevations in mRNA encoding IL-1β, TNF-α, IFN-γ, CD11b, IDO, and KMO were detected 24 h after kainic acid injection (Gleeson et al., 2010). Though correlative, it is plausible that these elevations in proinflammatory cytokines underlie the induction of IDO and KMO in this model since IL-1β, TNF-α, and IFN-γ are all potent inducers of IDO and at least IFN-γ also induces KMO expression as well (Mandi and Vecsei, 2012). While it may be surmised that induction of IDO and KMO likely leads to central enhancement in 3-HK and QUIN production in this model, it is not at all clear what, if any, role these metabolites might play in either acute seizure activity or in epileptogenesis. It is reasonable to hypothesize that the pro-convulsant activity of QUIN may at least exacerbate neuronal hyperactivity and/or excitotoxicity. Furthermore, both QUIN and 3-HK may contribute to neuronal degeneration to further aggravate the neuroinflammatory responses that underlie or contribute to disease pathology. To answer such questions should be relatively straightforward with the availability of molecular, genetic, and pharmacological tools to dissect the relationship between inflammatory cytokine signaling and KP metabolism in the context of epilepsy.

Potential therapeutic intervention by modulation of kynurenine pathway in epilepsy

While there is little clinical evidence to date supporting the notion that KP metabolism is dysregulated in epilepsy, this possibility is strengthened by our emerging understanding of the role neuroinflammation may play in the precipitation and recurrence of epileptic seizure activity, combined with the regulation of KP activity by proinflammatory cytokine signaling. Based on this and recent pre-clinical data (Lehrmann et al., 2008; Gleeson et al., 2010), we may predict that the microglial branch is overactive with respect to the astrocytic branch of the KP in at least some forms of epilepsy, resulting in excessive accumulation of 3-HK and QUIN in the CNS. If 3-HK and QUIN-mediated excitotoxicity or neurodegeneration do indeed contribute to disease pathology, then chronic, adjunctive treatment with a centrally penetrant KMO inhibitor might improve long term outcome compared to treatment with standard anticonvulsants alone, since KMO inhibition is proposed to increase the production of KYNA while decreasing the production of 3-HK and QUIN in the CNS,

DEPRESSION AND MAJOR DEPRESSIVE DISORDER

Depression is the most prevalent neuropsychological disorder. Worldwide figures estimate that ∼20% of people will experience a major depressive episode throughout the course of their lifetime (Kessler et al., 2005). Understanding the etiology of major depressive disorder (MDD) is complicated by sociodemographic factors and polygenetic contributions. Emerging data show that dysregulation of the immune system, over expression of proinflammatory cytokines, and aberrant tryptophan metabolism are contributing factors at least in a subset of MDD cases.

Role of inflammation and kynurenine metabolism in depression from clinical and human tissue studies

Clinical evidence for an inflammation component in MDD is quite strong. The most direct argument for a causative link stems from studies in which immune stimulating agents induce depressive symptoms in patients and/or healthy subjects. A common therapy for treating hepatitis C is the use of IFN-α. Up to 50% of these patients develop depressive symptoms that are maintained throughout the course of treatment but subside within a short period after completion (Bonaccorso et al., 2002a,b). Of interest within these patients, IFN-α treatment can enhance tryptophan metabolism through the KP pathway as measured by K/T ratios, an indicator of IDO activity (Capuron et al., 2003). Tryptophan was typically reduced in serum samples, though not always (Comai et al., 2011), and kynurenine levels increased during IFN-α treatment. The alteration in K/T ratios correlated with symptoms of depression and anxiety scores on the Montgomery–Åsberg Depression Rating Scale (MADRS), Beck Depression Inventory (BDI), and Hamilton Anxiety Rating Scale (HAM-A), respectively (Bonaccorso et al., 2002b). When evaluated using the BDI scale all hepatitis C patients treated with IFN-α showed worsening scores as well as increased K/T ratios. However, only a subset (26/45) reached the criteria to be considered depressed. Interestingly, this patient subset also showed the greatest disruption in tryptophan metabolism and highest K/T ratios.

Similar to the results reported in hepatitis C populations, cancer patients treated with IFN-α also increase production of kynurenine and often possess lower tryptophan levels. In these patients K/T ratios were elevated and appeared to correlate with worsening outcome of cancer as well as development of depressive symptoms (Kurz et al., 2011). However, Bannink et al. (2007) showed that cancer patients treated with IFN-α had higher K/T ratios but did not develop symptoms of depression. It is worth noting that patients with a history of depression were excluded from this trial and as such, they may have been measuring *de novo* depression in a symptom resistant population. This interpretation would seem in line with the findings in hepatitis C

patients where only a subset of patients develop depression, potentially correlating with an underlying susceptibility (Comai et al., 2011). Furthermore, the relationship between K/T ratios and depressive symptoms in melanoma patients treated with IFN-α and paroxetine supports this hypothesis. IFN-α increased K/T ratios in melanoma patients and produced depressive symptoms (Capuron et al., 2003). Paroxetine reduced the depressive symptoms and increased tryptophan, without affecting kynurenine, resulting in only modestly elevated K/T ratios. Alternatively, in patients not receiving an antidepressant, tryptophan levels were lower and kynurenine levels higher in those who developed depression compared to more resilient patients.

In healthy volunteers, stimulation of the immune system causes increased proinflammatory cytokine production and increased kynurenine production associated with depressive symptoms (Eisenberger et al., 2010). Low doses of endotoxin (from *E. coli*) increased TNF-α, IL-6, and body temperature. Interestingly, induction of the immune response correlated with reduced ventral striatum activation in a monetary incentive task, suggesting reduced function of reward systems. In other studies, higher plasma K/T ratios correlated with anhedonia scores in adolescents with MDD (Gabbay et al., 2012). Furthermore, in children with melancholic MDD, K/T, kynurenine, and 3-HAA/L-KYN levels were associated with severity of depressive symptoms (Gabbay et al., 2010).

Though a broad range of clinical studies support a role for inflammation-mediated dysregulation of cytokine production and kynurenine metabolism in MDD, some studies demonstrate a lack of correlation between inflammation, K/T ratios, and depressive symptoms. In one case, plasma IL-6 levels were reported along with a minor increase in IFN-α in a depressed cohort (Hughes et al., 2012). No evidence of increased kynurenine metabolism was observed though tryptophan was decreased. These patients also possessed elevated C-reactive protein (CRP) levels (2.1 mg/L vs. 1.2 mg/L in controls), typically used as an indicator of underlying inflammation, though they remained within a normal range. These data support the hypothesis that tryptophan depletion occurs independent of kynurenine metabolism by IDO in patients with minimal inflammation. Indeed, anti-inflammatory therapies have been found to be effective at treating depression in patients with high levels of CRP (>5 mg/L) (Raison et al., 2013). Furthermore, where it has been evaluated, proinflammatory markers such as IL-1β, TNF-α, and macrophage migration inhibitory factor appear to predict lack of responsiveness to traditional antidepressant medications (Cattaneo et al., 2013). In addition, levels of tryptophan, kynurenine, and 3-HAA correlated to treatment response to fluoxetine across a broad range of clinical scales (Mackay et al., 2009). Together these data suggest that only a subset of MDD patients with high levels of underlying inflammation are associated with disruption in kynurenine metabolism that relates to depressive symptoms.

A genetic link between inflammation and kynurenine metabolism in MDD was reported in patients with IFN-γ (+874) T/A genotypes. Healthy women with the higher IFN-γ producing T allele were associated with increased IDO activity as measured by elevated plasma levels of K/T compared to the lower producing A allele (Raitala et al., 2005). In addition, TA carriers had a higher prevalence of depression than the AA genotype (Oxenkrug et al., 2011). More recently, an IFN-γ CA repeat polymorphism was identified that also conferred lower tryptophan levels along with higher kynurenine production (Myint et al., 2013), though the relationship between symptoms of depression and kynurenine metabolism have yet to be evaluated in these patients. Furthermore, a polymorphism in the promoter region of the gene for IDO correlated with increased depression in hepatitis C patients treated with IFN-α (Smith et al., 2012). In the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial two common SNPs in the IDO1 gene were associated with treatment outcome for either citalopram or overall antidepressant treatment (Cutler et al., 2012).

Though upregulated kynurenine production in serum is a relatively common finding in MDD studies, fewer reports have evaluated neuroinflammation in this disorder. QUIN is elevated in the anterior cingulate cortex of depressed patients, but only in severely depressed individuals (Steiner et al., 2011). In addition, studies have now demonstrated that, along with increased plasma kynurenine (Sublette et al., 2011), QUIN and IL-6 are increased in the cerebrospinal fluid of suicide attempters (Erhardt et al., 2013). Intriguingly, the correlation between over activation of the QUIN branch of the KP in suicide attempters was confirmed in patients with a diagnosis other than MDD as well. These data suggest that in addition to inflammation-mediated IDO activation peripherally, and perhaps within the CNS, selective metabolism of kynurenine along the QUIN branch occurs in the brains of severely depressed patients.

Delineation of the role of inflammation on kynurenine metabolism and depressive symptoms in preclinical systems

Preclinical studies strongly support the link between immune stimulation, induction of kynurenine metabolism, and development of depressive-like symptoms (Dantzer et al., 2011; Leonard and Maes, 2012). Acute application of an immune stimulus such as LPS induces expression of IDO, IFN-γ, TNF-α, and IL-1β in animals (O'connor et al., 2009c) while also causing impairment in forced swim (FST) and tail suspension (TST) tests, assays measuring depressive-like behavior. Blockade of IDO with 1-MT prevented the induction of IDO, attenuated increased K/T in the brain and periphery, and alleviated behavioral impairments. Interestingly IFN-γ, TNF-α, and IL-1β remained elevated suggesting that these responses to LPS occurred upstream of IDO induction. Similarly, mice treated with LPS developed an anhedonic phenotype measured by sucrose or saccharine preference which was also blocked by IDO inhibition (Salazar et al., 2012). While LPS induces sickness-like behavior which may confound the measurement of depressive-like responses in animal models, most studies demonstrate that the sickness is more transient, allowing measurement of depressive-like behavior once sickness has subsided. In fractalkine-deficient mice ($CX3CR1^{-/-}$), chronic treatment with 1-MT prevented depressive symptoms precipitated by LPS for up to 72 h, though inhibiting IDO had no effect on sickness behavior which abated between 24 and 48 h (Corona et al., 2013).

Infusion of LPS intracerebroventricularly (icv) is used as a model of acute neuroinflammation to study the effects of cytokine regulation and depressive phenotypes in rodents. Local neuroinflammation increased kynurenine production and K/T ratios in both the CNS and in the periphery (Dobos et al., 2012). Furthermore, animals performed poorly in FST, though surprisingly no effect was observed in the elevated plus maze or in spontaneous alternation suggesting a lack of pro-anxiety responses or cognitive impairment. Inhibition of IDO with 1-MT prevented elevation of K/T as well as reduced immobility in the FST, suggesting that increased kynurenine production contributed to the depression-like phenotype. In addition to kynurenine dysregulation, icv LPS increased expression of IDO, TNF-α, IL-6, and iNOS mRNA in the brain (Fu et al., 2010). When tested acutely (4–8 h post-dose) animals also displayed significant reductions in social interaction, though it's worth noting that such an acute time period may be confounded by sickness behavior.

An alternative proinflammatory stimulus used to induce acute depressive-like responses is activation of TLR3 by Poly I:C, a synthetic dsRNA. Poly I:C induced a neuroinflammatory response characterized by transiently (<24 h) increased expression of TNF-α, IL-1β, and IL-6 with delayed increase in CD11b mRNA (24–28 h) in the frontal cortex and hippocampus of rats (Gibney et al., 2013). Depressive-like behaviors measured by saccharin preference and anxiogenic effects observed in the elevated plus maze after poly I:C treatment peaked at 48 h and persisted up to 72 h. Concurrent with the depressive phenotype, IDO expression along with tryptophan and kynurenine concentrations were elevated in the brain while no effect on 5-HT was observed. These data suggest that depressive phenotypes induced by viral-mimetic inflammation may be driven in part through dysregulation of the kynurenine system.

Chronic inflammatory stimuli also produce long-lasting depressive phenotypes associated with neuroinflammation and kynurenine dysregulation. BCG, an attenuated mycobacterium, induced an acute sickness period in mice lasting up to 5 days followed by a more prolonged depression-like phase that was sustained for weeks (Moreau et al., 2008). In this same model, kynurenine levels were increased for up to 3 weeks within the brain (Moreau et al., 2005). Dissection of the mechanism by which BCG regulates kynurenine metabolism and produces a depressive phenotype demonstrated that brain IDO, IFN-γ, and TNF-α are upregulated in concordance with depressive-like behavior. The depressive phenotype and kynurenine dysregulation produced by BCG inoculation was absent in IDO and IFN-γ KO mice (O'connor et al., 2009a,b). Furthermore, proinflammatory cytokines remained elevated in IDO KO mice, and to a lesser extent IFN-γ KO mice (e.g., IL-1β), suggesting that the impact on depression-like behaviors of cytokine induction subsequent to BCG treatment occurred through a downstream effect on kynurenine metabolism.

Both clinical and preclinical data support a link between neuroinflammation, kynurenine metabolism, and symptoms of depression. Induction of IDO, KMO, and KYNU by proinflammatory cytokines which cause disruption of normal physiological metabolism of tryptophan and/or kynurenine appears to be an important link in the cascade of events leading to certain forms of depression. Where tested in animal models, blockade of this induction has been beneficial in reversing or preventing development of depressive phenotypes. Though limited tools are available for testing the clinical benefit of manipulating the KP, it will be important for current (e.g., IDO inhibitors for the treatment of cancer) and future studies to evaluate the emotional status of patients in a systematic way to better understand the therapeutic potential of this system in MDD.

SCHIZOPHRENIA AND RELATED DISORDERS

Schizophrenia is a complex neuropsychiatric disorder affecting approximately 1% of the world population, characterized by positive (delusions, hallucinations, thought disorder), negative (anhedonia, alogia, asociality) and cognitive (deficits in attention, executive function, and memory) symptom clusters, attributed to disturbances in dopaminergic, glutamatergic, and GABAergic neurotransmission (Harrison and Weinberger, 2005; Lewis et al., 2005). A leading hypothesis posits that NMDAR hypofunction is a key neurobiological mechanism underlying the core features of the disease, initially inspired by the observation that NMDAR open channel blockers, such as phencyclidine and ketamine, recapitulate a wide spectrum of schizophrenia symptoms in healthy subjects, and exacerbate those of schizophrenic patients [for further review, see Coyle (2012), Moghaddam and Javitt (2012)]. This, combined with the notion that KYNA is proposed to function as an endogenous antagonist of the obligatory NMDAR co-agonist site, has stimulated intense interest in the involvement of KYNA in schizophrenia. Supporting this possibility, elevated KYNA levels have been detected in CSF (Erhardt et al., 2001; Nilsson et al., 2005; Linderholm et al., 2012) and post-mortem prefrontal cortex (Schwarcz et al., 2001) of schizophrenic patients compared to controls. While human or rodent brain tissue levels of KYNA (nM range) are below the reported IC_{50} for the NMDAR co-agonist site (μM range), emerging mechanistic and behavioral data from animal studies are consistent with an impact of fluctuations in endogenous brain KYNA on schizophrenia-related phenotypes (Erhardt et al., 2009; Wonodi and Schwarcz, 2010), suggesting that local synaptic or extrasynaptic concentrations of KYNA might be much higher than the reported global levels.

Putative mechanisms underlying kynurenic acid dysregulation in schizophrenia and related disorders

Elevation in the level of brain KYNA may result from increased availability of L-KYN for metabolism by KAT II, the predominant KYNA-synthesizing enzyme in human and rat brain (Guidetti et al., 1997). One mechanism by which this may occur is through astrocyte-specific enhancement of L-KYN production, since brain KAT II is predominantly expressed in astrocytes compared to other neural cell types (Kiss et al., 2003; Guidetti et al., 2007b). Indeed, protein expression of TDO2 is selectively upregulated in white matter astrocytes of post-mortem frontal cortex of schizophrenic patients compared to that from control subjects, coincident with a significant elevation of TDO2 but not IDO mRNA levels (Miller et al., 2004). Similar results were obtained for post-mortem anterior cingulate cortex of subjects with schizophrenia and bipolar disorder, accompanied by an increase

in tissue levels of L-KYN compared to controls (Miller et al., 2006). Thus, selective upregulation of astrocytic TDO2-mediated L-KYN synthesis may partially account for the overproduction of KYNA in brain regions implicated in cognitive impairment associated with schizophrenia. Regulatory mechanisms governing astrocytic TDO2 expression are not well-understood, though it is worth noting that the regulatory region of the gene encoding both human and rat TDO2 contain at least two glucocorticoid response elements (GREs), and TDO2 mRNA is induced by dexamethasone in rat liver (Danesch et al., 1983, 1987; Comings et al., 1995). Given this, it is tempting to speculate that, unlike the microglial branch of the KP, activity of the KYNA-producing astrocytic branch may be positively regulated by anti-inflammatory, rather than by proinflammatory signaling. This is consistent with the enhancement of brain KYNA production following administration of the COX-2 inhibitor parecoxib in rat (Schwieler et al., 2006), though the mechanism underlying this effect is unknown.

Another mechanism by which L-KYN availability for KAT II-mediated metabolism may be increased is through suppression of KMO expression and/or enzyme activity. KMO exhibits a relatively high affinity for L-KYN compared to that of KAT II, and therefore exerts preferential control over the fate of L-KYN. Thus, reduction in KMO activity is expected to increase the availability of L-KYN for KAT II-mediated metabolism, an effect which has been demonstrated experimentally using the KMO inhibitor JM-6 (Zwilling et al., 2011). Recently it has been reported that a coding SNP within the human KMO gene is associated with reduced KMO mRNA expression and elevated CSF KYNA in bipolar patients with psychotic features during mania (Lavebratt et al., 2013). Moreover, an intronic SNP within the human KMO gene is associated with reduced KMO mRNA expression and impaired schizophrenia-related endophenotypes (Wonodi et al., 2011). Thus, disease-relevant genetic impairment of KMO expression/activity might play a contributing role in the overproduction of KYNA in schizophrenia and related psychiatric disorders. It remains to be seen, however, whether KMO expression/activity may be similarly influenced by dysregulated inflammatory signaling associated with these disorders. As discussed earlier, expression of both IDO and KMO is induced by proinflammatory cytokines such as IFN-γ. Conversely, IFN-γ-mediated IDO expression is inhibited by IL-4 and IL-13 (Musso et al., 1994; Chaves et al., 2001), though opposing results have been reported (Yadav et al., 2007). Since IDO and KMO expression appear to be positively regulated by similar mechanisms, it would be interesting to determine whether KMO expression is similarly inhibited by IL-4 and/or IL-13, and whether such inhibition leads to overproduction of central KYNA. Such a hypothesis might be relevant to a role of cytokine-mediated KP dysregulation in schizophrenia, since a recent study of 26 schizophrenic patients and 26 control subjects found that the ratios of serum IFN-γ/IL-4, IFN-γ/IL-10, IL-2/IL-4, and TNF-α/IL-4 were significantly reduced in schizophrenic patients compared to controls (Chiang et al., 2013). These data support an emerging, though controversial hypothesis which proposes that schizophrenia is associated with a subtle shift from the production of Th1 cytokines, such as IFN-γ, IL-2, and TNF-α, toward the production of Th2 cytokines, such as IL-4, IL-10, and IL-6 [reviewed in Muller et al. (2012)].

Potential therapeutic intervention by modulation of kynurenine pathway in schizophrenia

Given the hypothesis that excessive extracellular KYNA in the CNS, through its inhibitory action at NMDAR glycineB receptors and/or α7 nicotinic acetylcholine receptors, contributes to schizophrenia symptomatology, research efforts to evaluate the therapeutic potential of reducing KYNA have focused on inhibition of KAT II, the predominant KYNA synthesizing enzyme in human and rat brain (Guidetti et al., 1997). Mounting preclinical evidence suggest that KAT II-selective inhibitors produce a pro-cognitive effect in schizophrenia-relevant behavioral assays. Acute hippocampal application of S-ESBA, a first-generation, brain-impenetrant KAT II inhibitor, reduces extracellular KYNA by ∼30% and leads to improved performance in the Morris water maze, an effect that was opposed by co-administration of a physiologically relevant concentration of KYNA (Pocivavsek et al., 2011). Acute systemic administration of second-generation KAT II-selective inhibitors, such as the brain-penetrant PF-04859989 (Dounay et al., 2013) reduce central KYNA concentrations by ∼70%, improve performance in a rodent sustained attention task, and reverse ketamine-induced working memory deficits in rodent and monkeys (Abbott et al., 2010; Chapin et al., 2010; Horner et al., 2010). Thus, combined with clinical and post-mortem data indicating elevated brain KYNA levels associated with schizophrenia, these pre-clinical data support the therapeutic utility of KAT II inhibitors in treating cognitive impairments associated with schizophrenia, symptom domains that are poorly treated by current standards of care.

ATTENTION DEFICIT-HYPERACTIVITY DISORDER

Attention Deficit-Hyperactivity Disorder (ADHD) is the most commonly diagnosed psychiatric disorder in children and adolescents. Though estimates of prevalence vary widely, it is estimated that ∼6–8% of school aged children suffer from this disorder (Larson et al., 2011; Willcutt, 2012). Patients show striking neuropsychological performance deficits compared to peers within their age-group which tend to diminish in severity over time. This may relate to a developmental delay of cortical maturation (Shaw and Rabin, 2009). The etiology of this delay is unknown but it has been hypothesized that impaired glial supply of energy to support neuronal activity could contribute. Recent developments in the study of ADHD suggest that patients may possess minor imbalances in their immunological systems, as measured by increased serum levels of IFN-γ and IL-13, while also having reduced levels of 3-HK though normal levels of kynurenine (Oades et al., 2010b). The altered levels of proinflammatory cytokine production and kynurenine metabolism trended toward normalizing in medicated subjects relative to medication naïve patients. These findings may be consistent with early hypotheses regarding an imbalance in tryptophan metabolism in ADHD which suggested that patients produce excess serotonin, at least in peripheral compartments (Irwin et al., 1981). An impaired production of 3-HK was predicted to reflect reduced activation of microglia

and thus impaired neuronal pruning that could contribute to developmental delays.

While no study has looked directly at CNS cytokine and kynurenine profiles in ADHD, a few have attempted to define behavioral endophenotypes associated with these markers in serum. In one study it was demonstrated that levels of S100b were negatively correlated to oppositional and conduct disorder symptoms (Oades et al., 2010a). In this same study, an inverse relationship between S100b and IL-10/IL-16 was observed which was in contrast to findings in healthy children. A subsequent study reported that elevated IL-16 levels, along with reduced S100b, were strongly correlated with hyperactivity while IL-13 may be related to attentional capacity (Oades et al., 2010b). Tryptophan metabolism was not directly related to symptoms, though increased kynurenine as well as elevated IFN-γ (though reduced TNF-α) were associated with faster reaction times. Interestingly another study showed that shorter pregnancy and lower birth weight of ADHD patients, factors that are associated with severity of symptoms, have been linked to increased 3-HK and IFN-γ (Oades, 2011) which is only partially consistent with earlier reports of dysregulated cytokine production and kynurenine metabolism, where reduced 3-HK was found. While findings that alterations in peripheral cytokine and kynurenine systems are an interesting start, additional work to establish whether these results translate to changes in the CNS compartment are needed. Furthermore, a detailed analysis of cytokine levels and their relationship to kynurenine metabolism in the brain over the course of the disease may shed light on the contribution of this system to the developmental delay reported to occur in ADHD patients.

HIV-ASSOCIATED NEUROCOGNITIVE DISORDER

Human Immunodeficiency Virus (HIV) infection is a debilitating chronic disease that causes dramatic $CD4^+$ T-cell depletion resulting in immune response deficiency as well as chronic immune activation and inflammation responses. A strong case exists for an involvement of tryptophan metabolic disturbances in the pathology of HIV infection. Activation of tryptophan metabolism by IDO likely favors HIV persistence and exacerbation of disease progression through immune response suppression and generation of neurotoxic metabolites. Elevated circulating levels of IFN-γ and kynurenine metabolites are commonly found in HIV patients (Fuchs et al., 1990). QUIN is elevated in serum and CSF from HIV infected persons and levels are correlated with progression of neuropsychological impairment over the course of the disease (Heyes et al., 1991a). Indeed, patients with HIV-associated dementia were reported to possess levels of QUIN that are ∼20-fold greater than non-infected controls. Similar increases in QUIN are observed in primate models after retroviral exposure indicating a causative link between HIV infection and activation of kynurenine metabolism (Heyes et al., 1990). However, the consequence of kynurenine dysregulation by HIV and its role in disease progression or symptomatology is unknown. Excessive activation of IDO may result in localized depletion of tryptophan availability leading to impaired T-cell differentiation, thereby suppressing immune function. In addition, inflammation-mediated induction of KMO and KYNU favors production of 3-HK and QUIN from kynurenine. 3-HK is involved in reactive oxygen species generation and also decreases the number of $CD4^+$ T-cells in corneal allograph studies (Zaher et al., 2011) suggesting this neuroactive metabolite could further impair immune function after HIV infection.

The mechanism by which HIV stimulates IDO expression is not entirely clear as it has been proposed to be mediated by both IFN-γ dependent (Brown et al., 1991) and independent (Boasso et al., 2009; Maneglier et al., 2009) mechanisms in human macrophages and T-cells. To be clear, both IFN-γ levels and IDO activity are increased in HIV patients, and though IFN-γ can induce IDO, the correlation that both pathways are engaged does not necessarily indicate a causative link between these effects. Thus, while IFN-γ production, particularly from opportunistic infections, may contribute to IDO expression and tryptophan metabolism, HIV also appears to be able to stimulate kynurenine production via an interaction with CD4 receptors independent of IFN-γ. Elevated CSF kynurenine metabolism occurs independent of macrophage infiltration in simian AIDs models (Heyes et al., 1991b), suggesting that elevated QUIN is synthesized by local CNS production, possibly by microglia in response to peripheral immune/inflammation signals. Further complicating this interaction is the fact that HIV replication is enhanced by TNF-α, IFN-γ, and IL-1β, all acting through NF-κB. Since NF-κB also stimulates IDO, KMO, and KYNU, it is possible that proinflammatory cytokine signaling underlies a vicious cycle that promotes viral replication, tryptophan/kynurenine metabolism, and progression of dementia symptoms. It can thus be hypothesized that HIV infects immune cells including macrophages, T-cells, and microglia causing activation and subsequent release of proinflammatory cytokines and induction of tryptophan metabolizing enzymes. The resulting impairment in immune response could allow for opportunistic infections which further increase proinflammatory cytokine production supporting generation of 3-HK and QUIN throughout the body and brain. While the precipitating factors behind viral replication and kynurenine dysregulation may be similar, the neurocognitive dysfunction observed in HIV-associated neurocognitive disorder or dementia may be mediated in part by aberrant kynurenine metabolism in microglia within the brain in response to chronic production of proinflammatory cytokines, which one might speculate could be treated by inhibition of IDO, KMO, or KYNU.

THERAPEUTIC POTENTIAL AND IMMUNE INTERACTIONS BY THE KYNURENINE PATHWAY

The KP is uniquely positioned to regulate both the nervous and immune systems in disease states, which presents an interesting potential for drug discovery efforts but also potential risks of immunological responses. A large number of ligands targeting inhibition of kynurenine-related enzymes are available, but none have thus far advanced to clinical studies with the exception of IDO inhibitors for cancer. Decreasing production of neurotoxic metabolites such as 3-HK and QUIN with IDO, KMO, or KYNU inhibitors may reduce neuronal loss or atrophy in diseases like AD, PD, and HD, and may also offer promising therapy for MDD and HIV-associated neurocognitive disorder where neuroinflammation may contribute to the pathophysiology. Unfortunately, most ligands currently available have poor

drug-like properties and would require optimization in terms of their pharmacokinetic properties. Alternatively, elevating levels of KYNA, or delivering KYNA-mimetics to the brain, may offer a different option for neuroprotection. However, it's worth noting that while KYNA analogs would be predicted to reduce excitotoxicity, it is unclear whether they could reduce oxidative stress-mediated damage caused by excessive production of 3-HK and QUIN. Furthermore, increasing KYNA may lead to psychotomimetic effects including disruption of cognitive function, whereas decreasing KYNA may be a therapeutic option for treating schizophrenia. Manipulating kynurenine metabolism for MS should be approached cautiously because the direction of change in kynurenines seems to differ depending on the stage of the disease. Also, the variability in the time course of disease progression between individuals makes it difficult to predict the stage of disease.

Evidence is emerging suggesting that IDO and downstream tryptophan catabolites play an important role in modulating immune responses. Though a review of the impact of kynurenine metabolism on the peripheral immune system is beyond the scope of the current review, it is important to keep this function of the kynurenine system in mind when considering the therapeutic potential of this pathway. An important role of IDO in immunosuppression and tolerance has been demonstrated during pregnancy, autoimmunity, resistance to tumors, and tolerance to allografts (Mellor and Munn, 2004; Barth and Raghuraman, 2012; Ban et al., 2013). Thus, therapeutic interventions involving the KP must be approached cautiously to examine the potential consequence on immunomodulatory actions. Particular attention should be paid to patients with autoimmune diseases, given that IDO may act as a negative regulator of the immune system to counteract autoimmunity. Two non-mutually exclusive theories have been proposed about how IDO and/or the kynurenine metabolites regulate the immune system: (1) IDO catabolism of tryptophan depletes this critical amino acid which is important for T-cell proliferation and (2) downstream kynurenine metabolites themselves inhibit certain immune cells (Frumento et al., 2002). Elevated IDO expression and kynurenines can have immunosuppressive effects by inhibiting $CD4^+$ T-cell functions, inducing regulatory T-cells, and inhibiting Natural Killer (NK) cells (Mandi and Vecsei, 2012). Thus, the KP is directly implicated in excitatory and inhibitory neuronal communication, regulated by proinflammatory cytokines and immune signals, contributes to the production of oxidative stress, and regulates immune cell function/phenotypes, thereby positioning it to act as a key interface between the brain and immune systems. This dual role will be important to consider as the KP is mined for potential therapeutic agents to treat debilitating CNS disorders.

SUMMARY

Much has been learned in the thirty-some years since the first description of the KP in CNS disease. As detailed above, pathway dysregulations have been described in virtually all major CNS diseases. The pathway is sensitive to inflammatory signaling and its products have neuromodulatory properties. It has unique properties as an interface between the immune system and neuronal signaling. Inhibition of unique enzymes in this pathway has been employed to ameliorate symptomatologies in several animal models of CNS disease. Successful translation of these preclinical efforts into actual drugs might open the venue for novel therapeutic interventions in human CNS disease.

REFERENCES

Abbott, A., Roberts, B. M., Turner, L., Campbell, D. W., Schaffer, C. L., Campbell, B., et al. (2010). Inhibition of kynurenine aminotransferase II (KAT II) protects against ketamine-induced cognitive impairment and improves spatial working memory. *Soc. Neurosci. Abstr.* 472.18.

Acuna-Castroviejo, D., Tapias, V., Lopez, L. C., Doerrier, C., Camacho, E., Carrion, M. D., et al. (2011). Protective effects of synthetic kynurenines on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in mice. *Brain Res. Bull.* 85, 133–140. doi: 10.1016/j.brainresbull.2011.03.008

Agaugue, S., Perrin-Cocon, L., Coutant, F., Andre, P., and Lotteau, V. (2006). 1-Methyl-tryptophan can interfere with TLR signaling in dendritic cells independently of IDO activity. *J. Immunol.* 177, 2061–2071.

Akimoto, H., Yamada, A., and Takikawa, O. (2007). Up-regulation of the brain indoleamine 2,3-dioxygenase activity in a mouse model of Alzheimer's disease by systemic endotoxin challenge. *Int. Cong.* 1304, 357–361. doi: 10.1016/j.ics.2007.07.026

Alberati-Giani, D., Ricciardi-Castagnoli, P., Kohler, C., and Cesura, A. M. (1996). Regulation of the kynurenine metabolic pathway by interferon-gamma in murine cloned macrophages and microglial cells. *J. Neurochem.* 66, 996–1004. doi: 10.1046/j.1471-4159.1996.66030996.x

Alsadany, M. A., Shehata, H. H., Mohamad, M. I., and Mahfouz, R. G. (2013). Histone deacetylases enzyme, copper, and IL-8 levels in patients with Alzheimer's disease. *Am. J. Alzheimers. Dis. Other Demen.* 28, 54–61. doi: 10.1177/1533317512467680

Amirkhani, A., Rajda, C., Arvidsson, B., Bencsik, K., Boda, K., Seres, E., et al. (2005). Interferon-beta affects the tryptophan metabolism in multiple sclerosis patients. *Eur. J. Neurol.* 12, 625–631. doi: 10.1111/j.1468-1331.2005.01041.x

Amori, L., Guidetti, P., Pellicciari, R., Kajii, Y., and Schwarcz, R. (2009). On the relationship between the two branches of the kynurenine pathway in the rat brain *in vivo*. *J. Neurochem.* 109, 316–325. doi: 10.1111/j.1471-4159.2009.05893.x

Arends, Y. M., Duyckaerts, C., Rozemuller, J. M., Eikelenboom, P., and Hauw, J. J. (2000). Microglia, amyloid and dementia in alzheimer disease. A correlative study. *Neurobiol. Aging* 21, 39–47. doi: 10.1016/S0197-4580(00)00094-4

Babcock, T. A., and Carlin, J. M. (2000). Transcriptional activation of indoleamine dioxygenase by interleukin 1 and tumor necrosis factor alpha in interferon-treated epithelial cells. *Cytokine* 12, 588–594. doi: 10.1006/cyto.1999.0661

Ban, Y., Chang, Y., Dong, B., Kong, B., and Qu, X. (2013). Indoleamine 2,3-dioxygenase levels at the normal and recurrent spontaneous abortion fetal-maternal interface. *J. Int. Med. Res.* 41, 1135–1149. doi: 10.1177/0300060513487642

Bannink, M., Fekkes, D., Van Gool, A. R., Kruit, W. H., Sleijfer, S., Van Der Holt, B., et al. (2007). Interferon-alpha influences tryptophan metabolism without inducing psychiatric side effects. *Neuropsychobiology* 55, 225–231. doi: 10.1159/000108382

Barth, H., and Raghuraman, S. (2012). Persistent infectious diseases say - IDO. Role of indoleamine-2,3-dioxygenase in disease pathogenesis and implications for therapy. *Crit. Rev. Microbiol.* 40, 360–368. doi: 10.3109/1040841X.2012.742037

Beal, M. F., Ferrante, R. J., Swartz, K. J., and Kowall, N. W. (1991). Chronic quinolinic acid lesions in rats closely resemble Huntington's disease. *J. Neurosci.* 11, 1649–1659.

Beal, M. F., Matson, W. R., Storey, E., Milbury, P., Ryan, E. A., Ogawa, T., et al. (1992). Kynurenic acid concentrations are reduced in Huntington's disease cerebral cortex. *J. Neurol. Sci.* 108, 80–87. doi: 10.1016/0022-510X(92)90191-M

Beal, M. F., Matson, W. R., Swartz, K. J., Gamache, P. H., and Bird, E. D. (1990). Kynurenine pathway measurements in Huntington's disease striatum: evidence for reduced formation of kynurenic acid. *J. Neurochem.* 55, 1327–1339. doi: 10.1111/j.1471-4159.1990.tb03143.x

Bjorkqvist, M., Wild, E. J., Thiele, J., Silvestroni, A., Andre, R., Lahiri, N., et al. (2008). A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease. *J. Exp. Med.* 205, 1869–1877. doi: 10.1084/jem.20080178

Bluthe, R. M., Lestage, J., Rees, G., Bristow, A., and Dantzer, R. (2002). Dual effect of central injection of recombinant rat interleukin-4 on lipopolysaccharide-induced sickness behavior in rats. *Neuropsychopharmacology* 26, 86–93. doi: 10.1016/S0893-133X(01)00305-0

Boasso, A., Vaccari, M., Fuchs, D., Hardy, A. W., Tsai, W. P., Tryniszewska, E., et al. (2009). Combined effect of antiretroviral therapy and blockade of IDO in SIV-infected rhesus macaques. *J. Immunol.* 182, 4313–4320. doi: 10.4049/jimmunol.0803314

Bonaccorso, S., Marino, V., Biondi, M., Grimaldi, F., Ippoliti, F., and Maes, M. (2002a). Depression induced by treatment with interferon-alpha in patients affected by hepatitis C virus. *J. Affect. Disord.* 72, 237–241. doi: 10.1016/S0165-0327(02)00264-1

Bonaccorso, S., Marino, V., Puzella, A., Pasquini, M., Biondi, M., Artini, M., et al. (2002b). Increased depressive ratings in patients with hepatitis C receiving interferon-alpha-based immunotherapy are related to interferon-alpha-induced changes in the serotonergic system. *J. Clin. Psychopharmacol.* 22, 86–90. doi: 10.1097/00004714-200202000-00014

Bonda, D. J., Mailankot, M., Stone, J. G., Garrett, M. R., Staniszewska, M., Castellani, R. J., et al. (2010). Indoleamine 2,3-dioxygenase and 3-hydroxykynurenine modifications are found in the neuropathology of Alzheimer's disease. *Redox Rep.* 15, 161–168. doi: 10.1179/174329210X12650506623645

Brown, R. R., Ozaki, Y., Datta, S. P., Borden, E. C., Sondel, P. M., and Malone, D. G. (1991). Implications of interferon-induced tryptophan catabolism in cancer, auto-immune diseases and AIDS. *Adv. Exp. Med. Biol.* 294, 425–435. doi: 10.1007/978-1-4684-5952-4_39

Cagnin, A., Kassiou, M., Meikle, S. R., and Banati, R. B. (2006). *In vivo* evidence for microglial activation in neurodegenerative dementia. *Acta Neurol. Scand. Suppl.* 185, 107–114. doi: 10.1111/j.1600-0404.2006.00694.x

Campesan, S., Green, E. W., Breda, C., Sathyasaikumar, K. V., Muchowski, P. J., Schwarcz, R., et al. (2011). The kynurenine pathway modulates neurodegeneration in a Drosophila model of Huntington's disease. *Curr. Biol.* 21, 961–966. doi: 10.1016/j.cub.2011.04.028

Capuron, L., Neurauter, G., Musselman, D. L., Lawson, D. H., Nemeroff, C. B., Fuchs, D., et al. (2003). Interferon-alpha-induced changes in tryptophan metabolism. Relationship to depression and paroxetine treatment. *Biol. Psychiatry* 54, 906–914. doi: 10.1016/S0006-3223(03)00173-2

Carson, M. J. (2002). Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis. *Glia* 40, 218–231. doi: 10.1002/glia.10145

Cattaneo, A., Gennarelli, M., Uher, R., Breen, G., Farmer, A., Aitchison, K. J., et al. (2013). Candidate genes expression profile associated with antidepressants response in the GENDEP study: differentiating between baseline 'predictors' and longitudinal 'targets'. *Neuropsychopharmacology* 38, 377–385. doi: 10.1038/npp.2012.191

Chapin, D. S., Campbell, B., Strick, C., and Kozak, R. (2010). The impact of a KATII inhibitor on performance in the rat sustained attention task and conditioned avoidance responding. *Soc. Neurosci. Abstr.* 472.16.

Chaves, A. C., Ceravolo, I. P., Gomes, J. A., Zani, C. L., Romanha, A. J., and Gazzinelli, R. T. (2001). IL-4 and IL-13 regulate the induction of indoleamine 2,3-dioxygenase activity and the control of Toxoplasma gondii replication in human fibroblasts activated with IFN-gamma. *Eur. J. Immunol.* 31, 333–344. doi: 10.1002/1521-4141(200102)31:2<333::AID-IMMU333>3.0.CO;2-X

Chen, Y., and Guillemin, G. J. (2009). Kynurenine pathway metabolites in humans: disease and healthy states. *Int. J. Tryptophan Res.* 2, 1–19.

Chiang, S. S., Riedel, M., Schwarz, M., and Mueller, N. (2013). Is T-helper type 2 shift schizophrenia-specific? primary results from a comparison of related psychiatric disorders and healthy controls. *Psychiatry Clin. Neurosci.* 67, 228–236. doi: 10.1111/pcn.12040

Chiarugi, A., Calvani, M., Meli, E., Traggiai, E., and Moroni, F. (2001a). Synthesis and release of neurotoxic kynurenine metabolites by human monocyte-derived macrophages. *J. Neuroimmunol.* 120, 190–198. doi: 10.1016/S0165-5728(01)00418-0

Chiarugi, A., Cozzi, A., Ballerini, C., Massacesi, L., and Moroni, F. (2001b). Kynurenine 3-mono-oxygenase activity and neurotoxic kynurenine metabolites increase in the spinal cord of rats with experimental allergic encephalomyelitis. *Neuroscience* 102, 687–695. doi: 10.1016/S0306-4522(00)00504-2

Chon, S. Y., Hassanain, H. H., and Gupta, S. L. (1996). Cooperative role of interferon regulatory factor 1 and p91 (STAT1) response elements in interferon-gamma-inducible expression of human indoleamine 2,3-dioxygenase gene. *J. Biol. Chem.* 271, 17247–17252. doi: 10.1074/jbc.271.29.17247

Chon, S. Y., Hassanain, H. H., Pine, R., and Gupta, S. L. (1995). Involvement of two regulatory elements in interferon-gamma-regulated expression of human indoleamine 2,3-dioxygenase gene. *J. Interferon Cytokine Res.* 15, 517–526. doi: 10.1089/jir.1995.15.517

Comai, S., Cavalletto, L., Chemello, L., Bernardinello, E., Ragazzi, E., Costa, C. V., et al. (2011). Effects of PEG-interferon alpha plus ribavirin on tryptophan metabolism in patients with chronic hepatitis C. *Pharmacol. Res.* 63, 85–92. doi: 10.1016/j.phrs.2010.10.009

Comings, D. E., Muhleman, D., Dietz, G., Sherman, M., and Forest, G. L. (1995). Sequence of human tryptophan 2,3-dioxygenase (TDO2): presence of a glucocorticoid response-like element composed of a GTT repeat and an intronic CCCCT repeat. *Genomics* 29, 390–396. doi: 10.1006/geno.1995.9990

Connor, T. J., Starr, N., O'sullivan, J. B., and Harkin, A. (2008). Induction of indolamine 2,3-dioxygenase and kynurenine 3-monooxygenase in rat brain following a systemic inflammatory challenge: a role for IFN-gamma? *Neurosci. Lett.* 441, 29–34. doi: 10.1016/j.neulet.2008.06.007

Copeland, C. S., Neale, S. A., and Salt, T. E. (2013). Actions of Xanthurenic acid, a putative endogenous Group II metabotropic glutamate receptor agonist, on sensory transmission in the thalamus. *Neuropharmacology* 66, 133–142. doi: 10.1016/j.neuropharm.2012.03.009

Corona, A. W., Norden, D. M., Skendelas, J. P., Huang, Y., O'connor, J. C., Lawson, M., et al. (2013). Indoleamine 2,3-dioxygenase inhibition attenuates lipopolysaccharide induced persistent microglial activation and depressive-like complications in fractalkine receptor (CX(3)CR1)-deficient mice. *Brain Behav. Immun.* 31, 134–142. doi: 10.1016/j.bbi.2012.08.008

Coyle, J. T. (2012). NMDA receptor and schizophrenia: a brief history. *Schizophr. Bull.* 38, 920–926. doi: 10.1093/schbul/sbs076

Currier, A. R., Ziegler, M. H., Riley, M. M., Babcock, T. A., Telbis, V. P., and Carlin, J. M. (2000). Tumor necrosis factor-alpha and lipopolysaccharide enhance interferon-induced antichlamydial indoleamine dioxygenase activity independently. *J. Interferon Cytokine Res.* 20, 369–376. doi: 10.1089/107999000312306

Cutler, J. A., Rush, A. J., McMahon, F. J., and Laje, G. (2012). Common genetic variation in the indoleamine-2,3-dioxygenase genes and antidepressant treatment outcome in major depressive disorder. *J. Psychopharmacol.* 26, 360–367. doi: 10.1177/0269881111434622

Dai, W., and Gupta, S. L. (1990). Regulation of indoleamine 2,3-dioxygenase gene expression in human fibroblasts by interferon-gamma. Upstream control region discriminates between interferon-gamma and interferon-alpha. *J. Biol. Chem.* 265, 19871–19877.

Danesch, U., Gloss, B., Schmid, W., Schutz, G., Schule, R., and Renkawitz, R. (1987). Glucocorticoid induction of the rat tryptophan oxygenase gene is mediated by two widely separated glucocorticoid-responsive elements. *EMBO J.* 6, 625–630.

Danesch, U., Hashimoto, S., Renkawitz, R., and Schutz, G. (1983). Transcriptional regulation of the tryptophan oxygenase gene in rat liver by glucocorticoids. *J. Biol. Chem.* 258, 4750–4753.

Dantzer, R., O'connor, J. C., Lawson, M. A., and Kelley, K. W. (2011). Inflammation-associated depression: from serotonin to kynurenine. *Psychoneuroendocrinology* 36, 426–436. doi: 10.1016/j.psyneuen.2010.09.012

Darnell, J. E. Jr., Kerr, I. M., and Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264, 1415–1421. doi: 10.1126/science.8197455

Devinsky, O., Vezzani, A., Najjar, S., De Lanerolle, N. C., and Rogawski, M. A. (2013). Glia and epilepsy: excitability and inflammation. *Trends Neurosci.* 36, 174–184. doi: 10.1016/j.tins.2012.11.008

Dinatale, B. C., Murray, I. A., Schroeder, J. C., Flaveny, C. A., Lahoti, T. S., Laurenzana, E. M., et al. (2010). Kynurenic acid is a potent endogenous aryl hydrocarbon receptor ligand that synergistically induces interleukin-6 in the presence of inflammatory signaling. *Toxicol. Sci.* 115, 89–97. doi: 10.1093/toxsci/kfq024

Dobos, N., De Vries, E. F., Kema, I. P., Patas, K., Prins, M., Nijholt, I. M., et al. (2012). The role of indoleamine 2,3-dioxygenase in a mouse model of neuroinflammation-induced depression. *J. Alzheimers. Dis.* 28, 905–915. doi: 10.3233/JAD-2011-111097

Dounay, A. B., Anderson, M., Bechle, B. M., Evrard, E., Gan, X., Kim, J. Y., et al. (2013). PF-04859989 as a template for structure-based drug design:

identification of new pyrazole series of irreversible KAT II inhibitors with improved lipophilic efficiency. *Bioorg. Med. Chem. Lett.* 23, 1961–1966. doi: 10.1016/j.bmcl.2013.02.039

Durastanti, V., Lugaresi, A., Bramanti, P., Amato, M., Bellantonio, P., De Luca, G., et al. (2011). Neopterin production and tryptophan degradation during 24-months therapy with interferon beta-1a in multiple sclerosis patients. *J. Transl. Med.* 9:42. doi: 10.1186/1479-5876-9-42

Eisenberger, N. I., Inagaki, T. K., Mashal, N. M., and Irwin, M. R. (2010). Inflammation and social experience: an inflammatory challenge induces feelings of social disconnection in addition to depressed mood. *Brain Behav. Immun.* 24, 558–563. doi: 10.1016/j.bbi.2009.12.009

Erhardt, S., Blennow, K., Nordin, C., Skogh, E., Lindstrom, L. H., and Engberg, G. (2001). Kynurenic acid levels are elevated in the cerebrospinal fluid of patients with schizophrenia. *Neurosci. Lett.* 313, 96–98. doi: 10.1016/S0304-3940(01)02242-X

Erhardt, S., Lim, C. K., Linderholm, K. R., Janelidze, S., Lindqvist, D., Samuelsson, M., et al. (2013). Connecting inflammation with glutamate agonism in suicidality. *Neuropsychopharmacology* 38, 743–752. doi: 10.1038/npp.2012.248

Erhardt, S., Olsson, S. K., and Engberg, G. (2009). Pharmacological manipulation of kynurenic acid: potential in the treatment of psychiatric disorders. *CNS Drugs* 23, 91–101. doi: 10.2165/00023210-200923020-00001

Flanagan, E. M., Erickson, J. B., Viveros, O. H., Chang, S. Y., and Reinhard, J. F. Jr. (1995). Neurotoxin quinolinic acid is selectively elevated in spinal cords of rats with experimental allergic encephalomyelitis. *J. Neurochem.* 64, 1192–1196. doi: 10.1046/j.1471-4159.1995.64031192.x

Forrest, C. M., Mackay, G. M., Stoy, N., Spiden, S. L., Taylor, R., Stone, T. W., et al. (2010). Blood levels of kynurenines, interleukin-23 and soluble human leucocyte antigen-G at different stages of Huntington's disease. *J. Neurochem.* 112, 112–122. doi: 10.1111/j.1471-4159.2009.06442.x

Frumento, G., Rotondo, R., Tonetti, M., Damonte, G., Benatti, U., and Ferrara, G. B. (2002). Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *J. Exp. Med.* 196, 459–468. doi: 10.1084/jem.20020121

Fu, X., Zunich, S. M., O'connor, J. C., Kavelaars, A., Dantzer, R., and Kelley, K. W. (2010). Central administration of lipopolysaccharide induces depressive-like behavior *in vivo* and activates brain indoleamine 2,3 dioxygenase in murine organotypic hippocampal slice cultures. *J. Neuroinflammation* 7:43. doi: 10.1186/1742-2094-7-43

Fuchs, D., Moller, A. A., Reibnegger, G., Stockle, E., Werner, E. R., and Wachter, H. (1990). Decreased serum tryptophan in patients with HIV-1 infection correlates with increased serum neopterin and with neurologic/psychiatric symptoms. *J. Acquir. Immune Defic. Syndr.* 3, 873–876.

Fujigaki, H., Saito, K., Fujigaki, S., Takemura, M., Sudo, K., Ishiguro, H., et al. (2006). The signal transducer and activator of transcription 1alpha and interferon regulatory factor 1 are not essential for the induction of indoleamine 2,3-dioxygenase by lipopolysaccharide: involvement of p38 mitogen-activated protein kinase and nuclear factor-kappaB pathways, and synergistic effect of several proinflammatory cytokines. *J. Biochem.* 139, 655–662. doi: 10.1093/jb/mvj072

Fujigaki, H., Seishima, M., and Saito, K. (2012). Posttranslational modification of indoleamine 2,3-dioxygenase. *Anal. Bioanal. Chem.* 403, 1777–1782. doi: 10.1007/s00216-012-5946-2

Fujigaki, S., Saito, K., Sekikawa, K., Tone, S., Takikawa, O., Fujii, H., et al. (2001). Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism. *Eur. J. Immunol.* 31, 2313–2318. doi: 10.1002/1521-4141(200108)31:8%3C2313::AID-IMMU2313%3E3.0.CO;2-S

Fukui, S., Schwarcz, R., Rapoport, S. I., Takada, Y., and Smith, Q. R. (1991). Blood-brain barrier transport of kynurenines: implications for brain synthesis and metabolism. *J. Neurochem.* 56, 2007–2017. doi: 10.1111/j.1471-4159.1991.tb03460.x

Fuvesi, J., Rajda, C., Bencsik, K., Toldi, J., and Vecsei, L. (2012). The role of kynurenines in the pathomechanism of amyotrophic lateral sclerosis and multiple sclerosis: therapeutic implications. *J. Neural Transm.* 119, 225–234. doi: 10.1007/s00702-012-0765-3

Gabbay, V., Ely, B. A., Babb, J., and Liebes, L. (2012). The possible role of the kynurenine pathway in anhedonia in adolescents. *J. Neural Transm.* 119, 253–260. doi: 10.1007/s00702-011-0685-7

Gabbay, V., Liebes, L., Katz, Y., Liu, S., Mendoza, S., Babb, J. S., et al. (2010). The kynurenine pathway in adolescent depression: preliminary findings from a proton MR spectroscopy study. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 34, 37–44. doi: 10.1016/j.pnpbp.2009.09.015

Gal, E. M., and Sherman, A. D. (1978). Synthesis and metabolism of L-kynurenine in rat brain. *J. Neurochem.* 30, 607–613. doi: 10.1111/j.1471-4159.1978.tb07815.x

Gerhard, A., Pavese, N., Hotton, G., Turkheimer, F., Es, M., Hammers, A., et al. (2006). *In vivo* imaging of microglial activation with [11C](R)-PK11195 PET in idiopathic Parkinson's disease. *Neurobiol. Dis.* 21, 404–412. doi: 10.1016/j.nbd.2005.08.002

Gibney, S. M., McGuinness, B., Prendergast, C., Harkin, A., and Connor, T. J. (2013). Poly I:C-induced activation of the immune response is accompanied by depression and anxiety-like behaviours, kynurenine pathway activation and reduced BDNF expression. *Brain Behav. Immun.* 28, 170–181. doi: 10.1016/j.bbi.2012.11.010

Giorgini, F., Guidetti, P., Nguyen, Q., Bennett, S. C., and Muchowski, P. J. (2005). A genomic screen in yeast implicates kynurenine 3-monooxygenase as a therapeutic target for Huntington disease. *Nat. Genet.* 37, 526–531. doi: 10.1038/ng1542

Gitter, B. D., Cox, L. M., Rydel, R. E., and May, P. C. (1995). Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10738–10741. doi: 10.1073/pnas.92.23.10738

Gleeson, L. C., Ryan, K. J., Griffin, E. W., Connor, T. J., and Harkin, A. (2010). The beta2-adrenoceptor agonist clenbuterol elicits neuroprotective, anti-inflammatory and neurotrophic actions in the kainic acid model of excitotoxicity. *Brain Behav. Immun.* 24, 1354–1361. doi: 10.1016/j.bbi.2010.06.015

Goeb, J. L., Even, C., Nicolas, G., Gohier, B., Dubas, F., and Garre, J. B. (2006). Psychiatric side effects of interferon-beta in multiple sclerosis. *Eur. Psychiatry* 21, 186–193. doi: 10.1016/j.eurpsy.2005.09.013

Graham, W. C., Robertson, R. G., Sambrook, M. A., and Crossman, A. R. (1990). Injection of excitatory amino acid antagonists into the medial pallidal segment of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated primate reverses motor symptoms of parkinsonism. *Life Sci.* 47, PL91–PL97. doi: 10.1016/0024-3205(90)90376-3

Gregoire, L., Rassoulpour, A., Guidetti, P., Samadi, P., Bedard, P. J., Izzo, E., et al. (2008). Prolonged kynurenine 3-hydroxylase inhibition reduces development of levodopa-induced dyskinesias in parkinsonian monkeys. *Behav. Brain Res.* 186, 161–167. doi: 10.1016/j.bbr.2007.08.007

Guidetti, P., Amori, L., Sapko, M. T., Okuno, E., and Schwarcz, R. (2007a). Mitochondrial aspartate aminotransferase: a third kynurenate-producing enzyme in the mammalian brain. *J. Neurochem.* 102, 103–111. doi: 10.1111/j.1471-4159.2007.04556.x

Guidetti, P., Hoffman, G. E., Melendez-Ferro, M., Albuquerque, E. X., and Schwarcz, R. (2007b). Astrocytic localization of kynurenine aminotransferase II in the rat brain visualized by immunocytochemistry. *Glia* 55, 78–92. doi: 10.1002/glia.20432

Guidetti, P., Bates, G. P., Graham, R. K., Hayden, M. R., Leavitt, B. R., Macdonald, M. E., et al. (2006). Elevated brain 3-hydroxykynurenine and quinolinate levels in Huntington disease mice. *Neurobiol. Dis.* 23, 190–197. doi: 10.1016/j.nbd.2006.02.011

Guidetti, P., Luthi-Carter, R. E., Augood, S. J., and Schwarcz, R. (2004). Neostriatal and cortical quinolinate levels are increased in early grade Huntington's disease. *Neurobiol. Dis.* 17, 455–461. doi: 10.1016/j.nbd.2004.07.006

Guidetti, P., Okuno, E., and Schwarcz, R. (1997). Characterization of rat brain kynurenine aminotransferases I and II. *J. Neurosci. Res.* 50, 457–465. doi: 10.1002/(SICI)1097-4547(19971101)50:3<457::AID-JNR12>3.0.CO;2-3

Guidetti, P., Reddy, P. H., Tagle, D. A., and Schwarcz, R. (2000). Early kynurenergic impairment in Huntington's disease and in a transgenic animal model. *Neurosci. Lett.* 283, 233–235. doi: 10.1016/S0304-3940(00)00956-3

Guillemin, G. J., Kerr, S. J., Pemberton, L. A., Smith, D. G., Smythe, G. A., Armati, P. J., et al. (2001). IFN-beta1b induces kynurenine pathway metabolism in human macrophages: potential implications for multiple sclerosis treatment. *J. Interferon Cytokine Res.* 21, 1097–1101. doi: 10.1089/107999001317205231

Guillemin, G. J., Smythe, G. A., Veas, L. A., Takikawa, O., and Brew, B. J. (2003). A beta 1-42 induces production of quinolinic acid by human macrophages and microglia. *Neuroreport* 14, 2311–2315. doi: 10.1097/00001756-200312190-00005

Guillemin, G. J., Smythe, G., Takikawa, O., and Brew, B. J. (2005). Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons. *Glia* 49, 15–23. doi: 10.1002/glia.20090

Hamann, M., Sander, S. E., and Richter, A. (2008). Effects of the kynurenine 3-hydroxylase inhibitor Ro 61-8048 after intrastriatal injections on the severity of dystonia in the dt sz mutant. *Eur. J. Pharmacol.* 586, 156–159. doi: 10.1016/j.ejphar.2008.02.052

Haroon, E., Raison, C. L., and Miller, A. H. (2012). Psychoneuroimmunology meets neuropsychopharmacology: translational implications of the impact of inflammation on behavior. *Neuropsychopharmacology* 37, 137–162. doi: 10.1038/npp.2011.205

Harrison, P. J., and Weinberger, D. R. (2005). Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol. Psychiatry* 10, 40–68; image 45. doi: 10.1038/sj.mp.4001558

Hartai, Z., Klivenyi, P., Janaky, T., Penke, B., Dux, L., and Vecsei, L. (2005). Kynurenine metabolism in multiple sclerosis. *Acta Neurol. Scand.* 112, 93–96. doi: 10.1111/j.1600-0404.2005.00442.x

Hassanain, H. H., Chon, S. Y., and Gupta, S. L. (1993). Differential regulation of human indoleamine 2,3-dioxygenase gene expression by interferons-gamma and -alpha. Analysis of the regulatory region of the gene and identification of an interferon-gamma-inducible DNA-binding factor. *J. Biol. Chem.* 268, 5077–5084.

Heyes, M. P., Brew, B. J., Martin, A., Price, R. W., Salazar, A. M., Sidtis, J. J., et al. (1991a). Quinolinic acid in cerebrospinal fluid and serum in HIV-1 infection: relationship to clinical and neurological status. *Ann. Neurol.* 29, 202–209. doi: 10.1002/ana.410290215

Heyes, M. P., Lackner, A., Kaufman, S., and Milstien, S. (1991b). Cerebrospinal fluid and serum neopterin and biopterin in D-retrovirus-infected rhesus macaques (*Macaca mulatta*): relationship to clinical and viral status. *AIDS* 5, 555–560. doi: 10.1097/00002030-199105000-00012

Heyes, M. P., Chen, C. Y., Major, E. O., and Saito, K. (1997). Different kynurenine pathway enzymes limit quinolinic acid formation by various human cell types. *Biochem. J.* 326(Pt 2), 351–356.

Heyes, M. P., Mefford, I. N., Quearry, B. J., Dedhia, M., and Lackner, A. (1990). Increased ratio of quinolinic acid to kynurenic acid in cerebrospinal fluid of D retrovirus-infected rhesus macaques: relationship to clinical and viral status. *Ann. Neurol.* 27, 666–675. doi: 10.1002/ana.410270614

Heyes, M. P., Saito, K., and Markey, S. P. (1992). Human macrophages convert L-tryptophan into the neurotoxin quinolinic acid. *Biochem. J.* 283(Pt 3), 633–635.

Hissong, B. D., and Carlin, J. M. (1997). Potentiation of interferon-induced indoleamine 2,3-dioxygenase mRNA in human mononuclear phagocytes by lipopolysaccharide and interleukin-1. *J. Interferon Cytokine Res.* 17, 387–393. doi: 10.1089/jir.1997.17.387

Horner, W., Gorczyca, R. R., Hughes, Z., Campbell, B., Fonseca, K., and Verhoest, P. (2010). *In vivo* characterization of a potent, selective KATII inhibitor as a regulator of kynurenic acid production in rat brain: a microdialysis study in freely moving rats. *Soc. Neurosci. Abstr.* 472.4.

Hu, B., Hissong, B. D., and Carlin, J. M. (1995). Interleukin-1 enhances indoleamine 2,3-dioxygenase activity by increasing specific mRNA expression in human mononuclear phagocytes. *J. Interferon Cytokine Res.* 15, 617–624. doi: 10.1089/jir.1995.15.617

Huang, G., Shi, L. Z., and Chi, H. (2009). Regulation of JNK and p38 MAPK in the immune system: signal integration, propagation and termination. *Cytokine* 48, 161–169. doi: 10.1016/j.cyto.2009.08.002

Huang, Q., Zhou, D., Sapp, E., Aizawa, H., Ge, P., Bird, E. D., et al. (1995). Quinolinic acid-induced increases in calbindin D28k immunoreactivity in rat striatal neurons *in vivo* and *in vitro* mimic the pattern seen in Huntington's disease. *Neuroscience* 65, 397–407. doi: 10.1016/0306-4522(94)00494-P

Huell, M., Strauss, S., Volk, B., Berger, M., and Bauer, J. (1995). Interleukin-6 is present in early stages of plaque formation and is restricted to the brains of Alzheimer's disease patients. *Acta Neuropathol.* 89, 544–551. doi: 10.1007/BF00571510

Hughes, M. M., Carballedo, A., McLoughlin, D. M., Amico, F., Harkin, A., Frodl, T., et al. (2012). Tryptophan depletion in depressed patients occurs independent of kynurenine pathway activation. *Brain Behav. Immun.* 26, 979–987. doi: 10.1016/j.bbi.2012.05.010

Imamura, K., Hishikawa, N., Sawada, M., Nagatsu, T., Yoshida, M., and Hashizume, Y. (2003). Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. *Acta Neuropathol.* 106, 518–526. doi: 10.1007/s00401-003-0766-2

Irwin, M., Belendiuk, K., McCloskey, K., and Freedman, D. X. (1981). Tryptophan metabolism in children with attentional deficit disorder. *Am. J. Psychiatry* 138, 1082–1085.

Jansen, M., and Reinhard, J. F. Jr. (1999). Interferon response heterogeneity: activation of a proinflammatory response by interferon alpha and beta. A possible basis for diverse responses to interferon beta in MS. *J. Leukoc. Biol.* 65, 439–443.

Jauch, D., Urbanska, E. M., Guidetti, P., Bird, E. D., Vonsattel, J. P., Whetsell, W. O., et al. (1995). Dysfunction of brain kynurenic acid metabolism in Huntington's disease: focus on kynurenine aminotransferases. *J. Neurol. Sci.* 130, 39–47. doi: 10.1016/0022-510X(94)00280-2

Jung, I. D., Lee, C. M., Jeong, Y. I., Lee, J. S., Park, W. S., Han, J., et al. (2007). Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon gamma in murine bone marrow derived dendritic cells. *FEBS Lett.* 581, 1449–1456. doi: 10.1016/j.febslet.2007.02.073

Jung, I. D., Lee, M. G., Chang, J. H., Lee, J. S., Jeong, Y. I., Lee, C. M., et al. (2009). Blockade of indoleamine 2,3-dioxygenase protects mice against lipopolysaccharide-induced endotoxin shock. *J. Immunol.* 182, 3146–3154. doi: 10.4049/jimmunol.0803104

Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., and Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Arch. Gen. Psychiatry* 62, 617–627. doi: 10.1001/archpsyc.62.6.617

Kiss, C., Ceresoli-Borroni, G., Guidetti, P., Zielke, C. L., Zielke, H. R., and Schwarcz, R. (2003). Kynurenate production by cultured human astrocytes. *J. Neural Transm.* 110, 1–14.

Kita, T., Morrison, P. F., Heyes, M. P., and Markey, S. P. (2002). Effects of systemic and central nervous system localized inflammation on the contributions of metabolic precursors to the L-kynurenine and quinolinic acid pools in brain. *J. Neurochem.* 82, 258–268. doi: 10.1046/j.1471-4159.2002.00955.x

Knyihar-Csillik, E., Chadaide, Z., Mihaly, A., Krisztin-Peva, B., Fenyo, R., and Vecsei, L. (2006). Effect of 6-hydroxydopamine treatment on kynurenine aminotransferase-I (KAT-I) immunoreactivity of neurons and glial cells in the rat substantia nigra. *Acta Neuropathol.* 112, 127–137. doi: 10.1007/s00401-006-0086-4

Knyihar-Csillik, E., Csillik, B., Pakaski, M., Krisztin-Peva, B., Dobo, E., Okuno, E., et al. (2004). Decreased expression of kynurenine aminotransferase-I (KAT-I) in the substantia nigra of mice after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment. *Neuroscience* 126, 899–914. doi: 10.1016/j.neuroscience.2004.04.043

Konan, K. V., and Taylor, M. W. (1996). Importance of the two interferon-stimulated response element (ISRE) sequences in the regulation of the human indoleamine 2,3-dioxygenase gene. *J. Biol. Chem.* 271, 19140–19145. doi: 10.1074/jbc.271.32.19140

Krakauer, T., and Oppenheim, J. J. (1993). IL-1 and tumor necrosis factor-alpha each up-regulate both the expression of IFN-gamma receptors and enhance IFN-gamma-induced HLA-DR expression on human monocytes and a human monocytic cell line (THP-1). *J. Immunol.* 150, 1205–1211.

Kurz, K., Schroecksnadel, S., Weiss, G., and Fuchs, D. (2011). Association between increased tryptophan degradation and depression in cancer patients. *Curr. Opin. Clin. Nutr. Metab. Care* 14, 49–56. doi: 10.1097/MCO.0b013e328340d849

Kwidzinski, E., Bunse, J., Aktas, O., Richter, D., Mutlu, L., Zipp, F., et al. (2005). Indolamine 2,3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. *FASEB J.* 19, 1347–1349. doi: 10.1096/fj.04-3228fje

Larson, K., Russ, S. A., Kahn, R. S., and Halfon, N. (2011). Patterns of comorbidity, functioning, and service use for US children with ADHD, 2007. *Pediatrics* 127, 462–470. doi: 10.1542/peds.2010-0165

Lassmann, H., and Ransohoff, R. M. (2004). The CD4-Th1 model for multiple sclerosis: a critical [correction of crucial] re-appraisal. *Trends Immunol.* 25, 132–137. doi: 10.1016/j.it.2004.01.007

Lavebratt, C., Olsson, S., Backlund, L., Frisen, L., Sellgren, C., Priebe, L., et al. (2013). The KMO allele encoding Arg is associated with psychotic features in bipolar disorder type 1, and with increased CSF KYNA level and reduced KMO expression. *Mol. Psychiatry*. doi: 10.1038/mp.2013.11. [Epub ahead of print].

Lee Do, Y., Lee, K. S., Lee, H. J., Noh, Y. H., Kim Do, H., Lee, J. Y., et al. (2008). Kynurenic acid attenuates MPP(+)-induced dopaminergic neuronal cell death via a Bax-mediated mitochondrial pathway. *Eur. J. Cell Biol.* 87, 389–397. doi: 10.1016/j.ejcb.2008.03.003

Lehrmann, E., Guidetti, P., Love, A., Williamson, J., Bertram, E. H., and Schwarcz, R. (2008). Glial activation precedes seizures and hippocampal

neurodegeneration in measles virus-infected mice. *Epilepsia* 49(Suppl. 2), 13–23. doi: 10.1111/j.1528-1167.2008.01489.x

Leonard, B., and Maes, M. (2012). Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. *Neurosci. Biobehav. Rev.* 36, 764–785. doi: 10.1016/j.neubiorev.2011.12.005

Lestage, J., Verrier, D., Palin, K., and Dantzer, R. (2002). The enzyme indoleamine 2,3-dioxygenase is induced in the mouse brain in response to peripheral administration of lipopolysaccharide and superantigen. *Brain Behav. Immun.* 16, 596–601. doi: 10.1016/S0889-1591(02)00014-4

Lewis, D. A., Hashimoto, T., and Volk, D. W. (2005). Cortical inhibitory neurons and schizophrenia. *Nat. Rev. Neurosci.* 6, 312–324. doi: 10.1038/nrn1648

Liimatainen, S., Lehtimaki, K., Raitala, A., Peltola, M., Oja, S. S., Peltola, J., et al. (2011). Increased indoleamine 2,3-dioxygenase (IDO) activity in idiopathic generalized epilepsy. *Epilepsy Res.* 94, 206–212. doi: 10.1016/j.eplepsyres.2011.02.003

Linderholm, K. R., Skogh, E., Olsson, S. K., Dahl, M. L., Holtze, M., Engberg, G., et al. (2012). Increased levels of kynurenine and kynurenic acid in the CSF of patients with schizophrenia. *Schizophr. Bull.* 38, 426–432. doi: 10.1093/schbul/sbq086

Luchowski, P., Luchowska, E., Turski, W. A., and Urbanska, E. M. (2002). 1-Methyl-4-phenylpyridinium and 3-nitropropionic acid diminish cortical synthesis of kynurenic acid via interference with kynurenine aminotransferases in rats. *Neurosci. Lett.* 330, 49–52. doi: 10.1016/S0304-3940(02)00735-8

Lue, L. F., Rydel, R., Brigham, E. F., Yang, L. B., Hampel, H., Murphy, G. M., et al. (2001). Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia *in vitro*. *Glia* 35, 72–79. doi: 10.1002/glia.1072

Macchiarulo, A., Camaioni, E., Nuti, R., and Pellicciari, R. (2009). Highlights at the gate of tryptophan catabolism: a review on the mechanisms of activation and regulation of indoleamine 2,3-dioxygenase (IDO), a novel target in cancer disease. *Amino Acids* 37, 219–229. doi: 10.1007/s00726-008-0137-3

Mackay, G. M., Forrest, C. M., Christofides, J., Bridel, M. A., Mitchell, S., Cowlard, R., et al. (2009). Kynurenine metabolites and inflammation markers in depressed patients treated with fluoxetine or counselling. *Clin. Exp. Pharmacol. Physiol.* 36, 425–435. doi: 10.1111/j.1440-1681.2008.05077.x

Mandi, Y., and Vecsei, L. (2012). The kynurenine system and immunoregulation. *J. Neural Transm.* 119, 197–209. doi: 10.1007/s00702-011-0681-y

Maneglier, B., Malleret, B., Guillemin, G. J., Spreux-Varoquaux, O., Devillier, P., Rogez-Kreuz, C., et al. (2009). Modulation of indoleamine-2,3-dioxygenase expression and activity by HIV-1 in human macrophages. *Fundam. Clin. Pharmacol.* 23, 573–581. doi: 10.1111/j.1472-8206.2009.00703.x

Matysiak, M., Stasiolek, M., Orlowski, W., Jurewicz, A., Janczar, S., Raine, C. S., et al. (2008). Stem cells ameliorate EAE via an indoleamine 2,3-dioxygenase (IDO) mechanism. *J. Neuroimmunol.* 193, 12–23. doi: 10.1016/j.jneuroim.2007.07.025

McGeer, P. L., Itagaki, S., Boyes, B. E., and McGeer, E. G. (1988). Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* 38, 1285–1291. doi: 10.1212/WNL.38.8.1285

Mellor, A. L., Baban, B., Chandler, P., Marshall, B., Jhaver, K., Hansen, A., et al. (2003). Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J. Immunol.* 171, 1652–1655.

Mellor, A. L., and Munn, D. H. (2004). IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* 4, 762–774. doi: 10.1038/nri1457

Mezrich, J. D., Fechner, J. H., Zhang, X., Johnson, B. P., Burlingham, W. J., and Bradfield, C. A. (2010). An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J. Immunol.* 185, 3190–3198. doi: 10.4049/jimmunol.0903670

Miller, C. L., Llenos, I. C., Dulay, J. R., Barillo, M. M., Yolken, R. H., and Weis, S. (2004). Expression of the kynurenine pathway enzyme tryptophan 2,3-dioxygenase is increased in the frontal cortex of individuals with schizophrenia. *Neurobiol. Dis.* 15, 618–629. doi: 10.1016/j.nbd.2003.12.015

Miller, C. L., Llenos, I. C., Dulay, J. R., and Weis, S. (2006). Upregulation of the initiating step of the kynurenine pathway in postmortem anterior cingulate cortex from individuals with schizophrenia and bipolar disorder. *Brain Res.* 1073–1074, 25–37. doi: 10.1016/j.brainres.2005.12.056

Miranda, A. F., Boegman, R. J., Beninger, R. J., and Jhamandas, K. (1997). Protection against quinolinic acid-mediated excitotoxicity in nigrostriatal dopaminergic neurons by endogenous kynurenic acid. *Neuroscience* 78, 967–975. doi: 10.1016/S0306-4522(96)00655-0

Moghaddam, B., and Javitt, D. (2012). From revolution to evolution: the glutamate hypothesis of schizophrenia and its implication for treatment. *Neuropsychopharmacology* 37, 4–15. doi: 10.1038/npp.2011.181

Mogi, M., Harada, M., Kondo, T., Riederer, P., Inagaki, H., Minami, M., et al. (1994a). Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from parkinsonian patients. *Neurosci. Lett.* 180, 147–150. doi: 10.1016/0304-3940(94)90508-8

Mogi, M., Harada, M., Riederer, P., Narabayashi, H., Fujita, K., and Nagatsu, T. (1994b). Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neurosci. Lett.* 165, 208–210. doi: 10.1016/0304-3940(94)90746-3

Möller, T. (2010). Neuroinflammation in Huntington's disease. *J. Neural Transm.* 117, 1001–1008. doi: 10.1007/s00702-010-0430-7

Molteni, R., Macchi, F., Zecchillo, C., Dell'agli, M., Colombo, E., Calabrese, F., et al. (2013). Modulation of the inflammatory response in rats chronically treated with the antidepressant agomelatine. *Eur. Neuropsychopharmacol.* 23, 1645–1655. doi: 10.1016/j.euroneuro.2013.03.008

Monaco, F., Fumero, S., Mondino, A., and Mutani, R. (1979). Plasma and cerebrospinal fluid tryptophan in multiple sclerosis and degenerative diseases. *J. Neurol. Neurosurg. Psychiatr.* 42, 640–641. doi: 10.1136/jnnp.42.7.640

Moreau, M., Andre, C., O'connor, J. C., Dumich, S. A., Woods, J. A., Kelley, K. W., et al. (2008). Inoculation of Bacillus Calmette-Guerin to mice induces an acute episode of sickness behavior followed by chronic depressive-like behavior. *Brain Behav. Immun.* 22, 1087–1095. doi: 10.1016/j.bbi.2008.04.001

Moreau, M., Lestage, J., Verrier, D., Mormede, C., Kelley, K. W., Dantzer, R., et al. (2005). Bacille Calmette-Guerin inoculation induces chronic activation of peripheral and brain indoleamine 2,3-dioxygenase in mice. *J. Infect. Dis.* 192, 537–544. doi: 10.1086/431603

Muller, N., Myint, A. M., and Schwarz, M. J. (2012). Inflammation in schizophrenia. *Adv. Protein Chem. Struct. Biol.* 88, 49–68. doi: 10.1016/B978-0-12-398314-5.00003-9

Munn, D. H., Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A., and Mellor, A. L. (1999). Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J. Exp. Med.* 189, 1363–1372. doi: 10.1084/jem.189.9.1363

Munn, D. H., Zhou, M., Attwood, J. T., Bondarev, I., Conway, S. J., Marshall, B., et al. (1998). Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 281, 1191–1193. doi: 10.1126/science.281.5380.1191

Musso, T., Gusella, G. L., Brooks, A., Longo, D. L., and Varesio, L. (1994). Interleukin-4 inhibits indoleamine 2,3-dioxygenase expression in human monocytes. *Blood* 83, 1408–1411.

Myint, A. M., Bondy, B., Baghai, T. C., Eser, D., Nothdurfter, C., Schule, C., et al. (2013). Tryptophan metabolism and immunogenetics in major depression: a role for interferon-gamma gene. *Brain Behav. Immun.* 31, 128–133. doi: 10.1016/j.bbi.2013.04.003

Nagatsu, T., Mogi, M., Ichinose, H., and Togari, A. (2000). Cytokines in Parkinson's disease. *J. Neural Transm. Suppl.* 58, 143–151. doi: 10.1007/978-3-7091-6301-6_19

Nguyen, N. T., Kimura, A., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., et al. (2010). Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 107, 19961–19966. doi: 10.1073/pnas.1014465107

Nilsson, L. K., Linderholm, K. R., Engberg, G., Paulson, L., Blennow, K., Lindstrom, L. H., et al. (2005). Elevated levels of kynurenic acid in the cerebrospinal fluid of male patients with schizophrenia. *Schizophr. Res.* 80, 315–322. doi: 10.1016/j.schres.2005.07.013

Niranjan, R. (2013). Molecular basis of etiological implications in Alzheimer's Disease: focus on neuroinflammation. *Mol. Neurobiol.* 48, 412–428. doi: 10.1007/s12035-013-8428-4

Oades, R. D. (2011). An exploration of the associations of pregnancy and perinatal features with cytokines and tryptophan/kynurenine metabolism in children with attention-deficit hyperactivity disorder (ADHD). *Atten. Defic. Hyperact. Disord.* 3, 301–318. doi: 10.1007/s12402-011-0062-2

Oades, R. D., Dauvermann, M. R., Schimmelmann, B. G., Schwarz, M. J., and Myint, A. M. (2010a). Attention-deficit hyperactivity disorder (ADHD) and

glial integrity: S100B, cytokines and kynurenine metabolism–effects of medication. *Behav. Brain Funct.* 6:29. doi: 10.1186/1744-9081-6-29

Oades, R. D., Myint, A. M., Dauvermann, M. R., Schimmelmann, B. G., and Schwarz, M. J. (2010b). Attention-deficit hyperactivity disorder (ADHD) and glial integrity: an exploration of associations of cytokines and kynurenine metabolites with symptoms and attention. *Behav. Brain Funct.* 6:32. doi: 10.1186/1744-9081-6-32

O'connor, J. C., Andre, C., Wang, Y., Lawson, M. A., Szegedi, S. S., Lestage, J., et al. (2009a). Interferon-gamma and tumor necrosis factor-alpha mediate the upregulation of indoleamine 2,3-dioxygenase and the induction of depressive-like behavior in mice in response to bacillus Calmette-Guerin. *J. Neurosci.* 29, 4200–4209. doi: 10.1523/JNEUROSCI.5032-08.2009

O'connor, J. C., Lawson, M. A., Andre, C., Briley, E. M., Szegedi, S. S., Lestage, J., et al. (2009b). Induction of IDO by bacille Calmette-Guerin is responsible for development of murine depressive-like behavior. *J. Immunol.* 182, 3202–3212. doi: 10.4049/jimmunol.0802722

O'connor, J. C., Lawson, M. A., Andre, C., Moreau, M., Lestage, J., Castanon, N., et al. (2009c). Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. *Mol. Psychiatry* 14, 511–522. doi: 10.1038/sj.mp.4002148

Ogawa, T., Matson, W. R., Beal, M. F., Myers, R. H., Bird, E. D., Milbury, P., et al. (1992). Kynurenine pathway abnormalities in Parkinson's disease. *Neurology* 42, 1702–1706. doi: 10.1212/WNL.42.9.1702

Ohmori, Y., Schreiber, R. D., and Hamilton, T. A. (1997). Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J. Biol. Chem.* 272, 14899–14907. doi: 10.1074/jbc.272.23.14899

Opitz, C. A., Litzenburger, U. M., Sahm, F., Ott, M., Tritschler, I., Trump, S., et al. (2011). An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature* 478, 197–203. doi: 10.1038/nature10491

Ott, M., Demisch, L., Engelhardt, W., and Fischer, P. A. (1993). Interleukin-2, soluble interleukin-2-receptor, neopterin, L-tryptophan and beta 2-microglobulin levels in CSF and serum of patients with relapsing-remitting or chronic-progressive multiple sclerosis. *J. Neurol.* 241, 108–114. doi: 10.1007/BF00869773

Ouattara, B., Belkhir, S., Morissette, M., Dridi, M., Samadi, P., Gregoire, L., et al. (2009). Implication of NMDA receptors in the antidyskinetic activity of cabergoline, CI-1041, and Ro 61-8048 in MPTP monkeys with levodopa-induced dyskinesias. *J. Mol. Neurosci.* 38, 128–142. doi: 10.1007/s12031-008-9137-8

Oxenkrug, G., Perianayagam, M., Mikolich, D., Requintina, P., Shick, L., Ruthazer, R., et al. (2011). Interferon-gamma (+874) T/A genotypes and risk of IFN-alpha-induced depression. *J. Neural Transm.* 118, 271–274. doi: 10.1007/s00702-010-0525-1

Patel, N. S., Paris, D., Mathura, V., Quadros, A. N., Crawford, F. C., and Mullan, M. J. (2005). Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. *J. Neuroinflammation* 2:9. doi: 10.1186/1742-2094-2-9

Pearson, S. J., and Reynolds, G. P. (1992). Increased brain concentrations of a neurotoxin, 3-hydroxykynurenine, in Huntington's disease. *Neurosci. Lett.* 144, 199–201. doi: 10.1016/0304-3940(92)90749-W

Perkins, M. N., and Stone, T. W. (1985). Actions of kynurenic acid and quinolinic acid in the rat hippocampus *in vivo*. *Exp. Neurol.* 88, 570–579. doi: 10.1016/0014-4886(85)90072-X

Petermann, F., and Korn, T. (2011). Cytokines and effector T cell subsets causing autoimmune CNS disease. *FEBS Lett.* 585, 3747–3757. doi: 10.1016/j.febslet.2011.03.064

Platten, M., Ho, P. P., Youssef, S., Fontoura, P., Garren, H., Hur, E. M., et al. (2005). Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. *Science* 310, 850–855. doi: 10.1126/science.1117634

Pocivavsek, A., Wu, H. Q., Potter, M. C., Elmer, G. I., Pellicciari, R., and Schwarcz, R. (2011). Fluctuations in endogenous kynurenic acid control hippocampal glutamate and memory. *Neuropsychopharmacology* 36, 2357–2367. doi: 10.1038/npp.2011.127

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., et al. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453, 65–71. doi: 10.1038/nature06880

Quintana, F. J., Murugaiyan, G., Farez, M. F., Mitsdoerffer, M., Tukpah, A. M., Burns, E. J., et al. (2010). An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20768–20773. doi: 10.1073/pnas.1009201107

Rahman, A., Ting, K., Cullen, K. M., Braidy, N., Brew, B. J., and Guillemin, G. J. (2009). The excitotoxin quinolinic acid induces tau phosphorylation in human neurons. *PLoS ONE* 4:e6344. doi: 10.1371/journal.pone.0006344

Raison, C. L., Rutherford, R. E., Woolwine, B. J., Shuo, C., Schettler, P., Drake, D. F., et al. (2013). A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. *JAMA psychiatry* 70, 31–41. doi: 10.1001/2013.jamapsychiatry.4

Raitala, A., Pertovaara, M., Karjalainen, J., Oja, S. S., and Hurme, M. (2005). Association of interferon-gamma +874(T/A) single nucleotide polymorphism with the rate of tryptophan catabolism in healthy individuals. *Scand. J. Immunol.* 61, 387–390. doi: 10.1111/j.1365-3083.2005.01586.x

Rejdak, K., Bartosik-Psujek, H., Dobosz, B., Kocki, T., Grieb, P., Giovannoni, G., et al. (2002). Decreased level of kynurenic acid in cerebrospinal fluid of relapsing-onset multiple sclerosis patients. *Neurosci. Lett.* 331, 63–65. doi: 10.1016/S0304-3940(02)00710-3

Rejdak, K., Petzold, A., Kocki, T., Kurzepa, J., Grieb, P., Turski, W. A., et al. (2007). Astrocytic activation in relation to inflammatory markers during clinical exacerbation of relapsing-remitting multiple sclerosis. *J. Neural Transm.* 114, 1011–1015. doi: 10.1007/s00702-007-0667-y

Richter, A., and Hamann, M. (2003). The kynurenine 3-hydroxylase inhibitor Ro 61-8048 improves dystonia in a genetic model of paroxysmal dyskinesia. *Eur. J. Pharmacol.* 478, 47–52. doi: 10.1016/j.ejphar.2003.08.038

Robinson, C. M., Hale, P. T., and Carlin, J. M. (2006). NF-kappa B activation contributes to indoleamine dioxygenase transcriptional synergy induced by IFN-gamma and tumor necrosis factor-alpha. *Cytokine* 35, 53–61. doi: 10.1016/j.cyto.2006.07.007

Robinson, C. M., Shirey, K. A., and Carlin, J. M. (2003). Synergistic transcriptional activation of indoleamine dioxygenase by IFN-gamma and tumor necrosis factor-alpha. *J. Interferon Cytokine Res.* 23, 413–421. doi: 10.1089/107999003322277829

Sakurai, K., Zou, J. P., Tschetter, J. R., Ward, J. M., and Shearer, G. M. (2002). Effect of indoleamine 2,3-dioxygenase on induction of experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 129, 186–196. doi: 10.1016/S0165-5728(02)00176-5

Salazar, A., Gonzalez-Rivera, B. L., Redus, L., Parrott, J. M., and O'connor, J. C. (2012). Indoleamine 2,3-dioxygenase mediates anhedonia and anxiety-like behaviors caused by peripheral lipopolysaccharide immune challenge. *Horm. Behav.* 62, 202–209. doi: 10.1016/j.yhbeh.2012.03.010

Samadi, P., Gregoire, L., Rassoulpour, A., Guidetti, P., Izzo, E., Schwarcz, R., et al. (2005). Effect of kynurenine 3-hydroxylase inhibition on the dyskinetic and antiparkinsonian responses to levodopa in Parkinsonian monkeys. *Mov. Disord.* 20, 792–802. doi: 10.1002/mds.20596

Sathyasaikumar, K. V., Stachowski, E. K., Amori, L., Guidetti, P., Muchowski, P. J., and Schwarcz, R. (2010). Dysfunctional kynurenine pathway metabolism in the R6/2 mouse model of Huntington's disease. *J. Neurochem.* 113, 1416–1425. doi: 10.1111/j.1471-4159.2010.06675.x

Schwarcz, R., Bruno, J. P., Muchowski, P. J., and Wu, H. Q. (2012). Kynurenines in the mammalian brain: when physiology meets pathology. *Nat. Rev. Neurosci.* 13, 465–477. doi: 10.1038/nrn3257

Schwarcz, R., Okuno, E., White, R. J., Bird, E. D., and Whetsell, W. O. Jr. (1988). 3-Hydroxyanthranilate oxygenase activity is increased in the brains of Huntington disease victims. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4079–4081. doi: 10.1073/pnas.85.11.4079

Schwarcz, R., and Pellicciari, R. (2002). Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. *J. Pharmacol. Exp. Ther.* 303, 1–10. doi: 10.1124/jpet.102.034439

Schwarcz, R., Rassoulpour, A., Wu, H. Q., Medoff, D., Tamminga, C. A., and Roberts, R. C. (2001). Increased cortical kynurenate content in schizophrenia. *Biol. Psychiatry* 50, 521–530. doi: 10.1016/S0006-3223(01)01078-2

Schwarcz, R., Speciale, C., and French, E. D. (1987). Hippocampal kynurenines as etiological factors in seizure disorders. *Pol. J. Pharmacol. Pharm.* 39, 485–494.

Schwieler, L., Erhardt, S., Nilsson, L., Linderholm, K., and Engberg, G. (2006). Effects of COX-1 and COX-2 inhibitors on the firing of rat midbrain dopaminergic neurons–possible involvement of endogenous kynurenic acid. *Synapse* 59, 290–298. doi: 10.1002/syn.20241

Shaw, P., and Rabin, C. (2009). New insights into attention-deficit/hyperactivity disorder using structural neuroimaging. *Curr. Psychiatry Rep.* 11, 393–398. doi: 10.1007/s11920-009-0059-0

Shimizu, T., Nomiyama, S., Hirata, F., and Hayaishi, O. (1978). Indoleamine 2,3-dioxygenase. Purification and some properties. *J. Biol. Chem.* 253, 4700–4706.

Shirey, K. A., Jung, J. Y., Maeder, G. S., and Carlin, J. M. (2006). Upregulation of IFN-gamma receptor expression by proinflammatory cytokines influences IDO activation in epithelial cells. *J. Interferon Cytokine Res.* 26, 53–62. doi: 10.1089/jir.2006.26.53

Silva-Adaya, D., Perez-De La Cruz, V., Villeda-Hernandez, J., Carrillo-Mora, P., Gonzalez-Herrera, I. G., Garcia, E., et al. (2011). Protective effect of L-kynurenine and probenecid on 6-hydroxydopamine-induced striatal toxicity in rats: implications of modulating kynurenate as a protective strategy. *Neurotoxicol. Teratol.* 33, 303–312. doi: 10.1016/j.ntt.2010.10.002

Silvestroni, A., Faull, R. L., Strand, A. D., and Moller, T. (2009). Distinct neuroinflammatory profile in post-mortem human Huntington's disease. *Neuroreport* 20, 1098–1103. doi: 10.1097/WNR.0b013e32832e34ee

Smith, A. K., Simon, J. S., Gustafson, E. L., Noviello, S., Cubells, J. F., Epstein, M. P., et al. (2012). Association of a polymorphism in the indoleamine-2,3-dioxygenase gene and interferon-alpha-induced depression in patients with chronic hepatitis C. *Mol. Psychiatry* 17, 781–789. doi: 10.1038/mp.2011.67

Steiner, J., Walter, M., Gos, T., Guillemin, G. J., Bernstein, H. G., Sarnyai, Z., et al. (2011). Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission? *J. Neuroinflammation* 8:94. doi: 10.1186/1742-2094-8-94

Stone, T. W., and Connick, J. H. (1985). Quinolinic acid and other kynurenines in the central nervous system. *Neuroscience* 15, 597–617. doi: 10.1016/0306-4522(85)90063-6

Stoy, N., Mackay, G. M., Forrest, C. M., Christofides, J., Egerton, M., Stone, T. W., et al. (2005). Tryptophan metabolism and oxidative stress in patients with Huntington's disease. *J. Neurochem.* 93, 611–623. doi: 10.1111/j.1471-4159.2005.03070.x

Sublette, M. E., Galfalvy, H. C., Fuchs, D., Lapidus, M., Grunebaum, M. F., Oquendo, M. A., et al. (2011). Plasma kynurenine levels are elevated in suicide attempters with major depressive disorder. *Brain Behav. Immun.* 25, 1272–1278. doi: 10.1016/j.bbi.2011.05.002

Suh, H. S., Zhao, M. L., Rivieccio, M., Choi, S., Connolly, E., Zhao, Y., et al. (2007). Astrocyte indoleamine 2,3-dioxygenase is induced by the TLR3 ligand poly(I:C): mechanism of induction and role in antiviral response. *J. Virol.* 81, 9838–9850. doi: 10.1128/JVI.00792-07

Takikawa, O., Kuroiwa, T., Yamazaki, F., and Kido, R. (1988). Mechanism of interferon-gamma action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-gamma and evaluation of the enzyme-mediated tryptophan degradation in its anticellular activity. *J. Biol. Chem.* 263, 2041–2048.

Tamim, M. K., Samadi, P., Morissette, M., Gregoire, L., Ouattara, B., Levesque, D., et al. (2010). Effect of non-dopaminergic drug treatment on Levodopa induced dyskinesias in MPTP monkeys: common implication of striatal neuropeptides. *Neuropharmacology* 58, 286–296. doi: 10.1016/j.neuropharm.2009.06.030

Tu, H., Rady, P. L., Juelich, T., Smith, E. M., Tyring, S. K., and Hughes, T. K. (2005). Cytokine regulation of tryptophan metabolism in the hypothalamic-pituitary-adrenal (HPA) axis: implications for protective and toxic consequences in neuroendocrine regulation. *Cell. Mol. Neurobiol.* 25, 673–680. doi: 10.1007/s10571-005-4007-1

Vecsei, L., Szalardy, L., Fulop, F., and Toldi, J. (2013). Kynurenines in the CNS: recent advances and new questions. *Nat. Rev. Drug Discov.* 12, 64–82. doi: 10.1038/nrd3793

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J. C., et al. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453, 106–109. doi: 10.1038/nature06881

Vezzani, A., Aronica, E., Mazarati, A., and Pittman, Q. J. (2013a). Epilepsy and brain inflammation. *Exp. Neurol.* 244, 11–21. doi: 10.1016/j.expneurol.2011.09.033

Vezzani, A., Friedman, A., and Dingledine, R. J. (2013b). The role of inflammation in epileptogenesis. *Neuropharmacology* 69, 16–24. doi: 10.1016/j.neuropharm.2012.04.004

Vogel, C. F., Goth, S. R., Dong, B., Pessah, I. N., and Matsumura, F. (2008). Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochem. Biophys. Res. Commun.* 375, 331–335. doi: 10.1016/j.bbrc.2008.07.156

Vondracek, J., Umannova, L., and Machala, M. (2011). Interactions of the aryl hydrocarbon receptor with inflammatory mediators: beyond CYP1A regulation. *Curr. Drug Metab* 12, 89–103. doi: 10.2174/138920011795016827

Walker, A. K., Budac, D. P., Bisulco, S., Lee, A. W., Smith, R. A., Beenders, B., et al. (2013). NMDA receptor blockade by ketamine abrogates lipopolysaccharide-induced depressive-like behavior in C57BL/6J mice. *Neuropsychopharmacology* 38, 1609–1616. doi: 10.1038/npp.2013.71

Wang, J., Simonavicius, N., Wu, X., Swaminath, G., Reagan, J., Tian, H., et al. (2006). Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35. *J. Biol. Chem.* 281, 22021–22028. doi: 10.1074/jbc.M603503200

Wang, Y., Lawson, M. A., Dantzer, R., and Kelley, K. W. (2010). LPS-induced indoleamine 2,3-dioxygenase is regulated in an interferon-gamma-independent manner by a JNK signaling pathway in primary murine microglia. *Brain Behav. Immun.* 24, 201–209. doi: 10.1016/j.bbi.2009.06.152

Widner, B., Leblhuber, F., and Fuchs, D. (2002). Increased neopterin production and tryptophan degradation in advanced Parkinson's disease. *J. Neural Transm.* 109, 181–189. doi: 10.1007/s007020200014

Widner, B., Sepp, N., Kowald, E., Kind, S., Schmuth, M., and Fuchs, D. (1999). Degradation of tryptophan in patients with systemic lupus erythematosus. *Adv. Exp. Med. Biol.* 467, 571–577. doi: 10.1007/978-1-4615-4709-9_71

Willcutt, E. G. (2012). The prevalence of DSM-IV attention-deficit/hyperactivity disorder: a meta-analytic review. *Neurotherapeutics* 9, 490–499. doi: 10.1007/s13311-012-0135-8

Wonodi, I., and Schwarcz, R. (2010). Cortical kynurenine pathway metabolism: a novel target for cognitive enhancement in Schizophrenia. *Schizophr. Bull.* 36, 211–218. doi: 10.1093/schbul/sbq002

Wonodi, I., Stine, O. C., Sathyasaikumar, K. V., Roberts, R. C., Mitchell, B. D., Hong, L. E., et al. (2011). Downregulated kynurenine 3-monooxygenase gene expression and enzyme activity in schizophrenia and genetic association with schizophrenia endophenotypes. *Arch. Gen. Psychiatry* 68, 665–674. doi: 10.1001/archgenpsychiatry.2011.71

Wu, W., Nicolazzo, J. A., Wen, L., Chung, R., Stankovic, R., Bao, S. S., et al. (2013). Expression of tryptophan 2,3-dioxygenase and production of kynurenine pathway metabolites in triple transgenic mice and human Alzheimer's disease brain. *PLoS ONE* 8:e59749. doi: 10.1371/journal.pone.0059749

Yadav, M. C., Burudi, E. M., Alirezaei, M., Flynn, C. C., Watry, D. D., Lanigan, C. M., et al. (2007). IFN-gamma-induced IDO and WRS expression in microglia is differentially regulated by IL-4. *Glia* 55, 1385–1396. doi: 10.1002/glia.20544

Yamada, A., Akimoto, H., Kagawa, S., Guillemin, G. J., and Takikawa, O. (2009). Proinflammatory cytokine interferon-gamma increases induction of indoleamine 2,3-dioxygenase in monocytic cells primed with amyloid beta peptide 1-42: implications for the pathogenesis of Alzheimer's disease. *J. Neurochem.* 110, 791–800. doi: 10.1111/j.1471-4159.2009.06175.x

Yan, Y., Zhang, G. X., Gran, B., Fallarino, F., Yu, S., Li, H., et al. (2010). IDO upregulates regulatory T cells via tryptophan catabolite and suppresses encephalitogenic T cell responses in experimental autoimmune encephalomyelitis. *J. Immunol.* 185, 5953–5961. doi: 10.4049/jimmunol.1001628

Yanagawa, Y., Iwabuchi, K., and Onoe, K. (2009). Co-operative action of interleukin-10 and interferon-gamma to regulate dendritic cell functions. *Immunology* 127, 345–353. doi: 10.1111/j.1365-2567.2008.02986.x

Zadori, D., Nyiri, G., Szonyi, A., Szatmari, I., Fulop, F., Toldi, J., et al. (2011). Neuroprotective effects of a novel kynurenic acid analogue in a transgenic mouse model of Huntington's disease. *J. Neural Transm.* 118, 865–875. doi: 10.1007/s00702-010-0573-6

Zaher, S. S., Germain, C., Fu, H., Larkin, D. F., and George, A. J. (2011). 3-hydroxykynurenine suppresses CD4+ T-cell proliferation, induces T-regulatory-cell development, and prolongs corneal allograft survival. *Invest. Ophthalmol. Vis. Sci.* 52, 2640–2648. doi: 10.1167/iovs.10-5793

Zinger, A., Barcia, C., Herrero, M. T., and Guillemin, G. J. (2011). The involvement of neuroinflammation and kynurenine pathway in Parkinson's disease. *Parkinsons. Dis.* 2011, 716859. doi: 10.4061/2011/716859

Zunszain, P. A., Anacker, C., Cattaneo, A., Choudhury, S., Musaelyan, K., Myint, A. M., et al. (2012). Interleukin-1beta: a new regulator of the kynurenine pathway affecting human hippocampal neurogenesis. *Neuropsychopharmacology* 37, 939–949. doi: 10.1038/npp.2011.277

Zwilling, D., Huang, S. Y., Sathyasaikumar, K. V., Notarangelo, F. M., Guidetti, P., Wu, H. Q., et al. (2011). Kynurenine 3-monooxygenase inhibition in blood ameliorates neurodegeneration. *Cell* 145, 863–874. doi: 10.1016/j.cell.2011.05.020

Conflict of Interest Statement: The authors are employees of Lundbeck Research USA.

Received: 25 October 2013; accepted: 20 January 2014; published online: 06 February 2014.

Citation: Campbell BM, Charych E, Lee AW and Möller T (2014) Kynurenines in CNS disease: regulation by inflammatory cytokines. Front. Neurosci. ***8****:12. doi: 10.3389/fnins.2014.00012*

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 17 January 2014
doi: 10.3389/fnins.2013.00271

Surgical manipulation compromises leukocyte mobilization responses and inflammation after experimental cerebral ischemia in mice

Adam Denes[1,2]*, Jesus M. Pradillo[1], Caroline Drake[1], Hannah Buggey[1], Nancy J. Rothwell[1] and Stuart M. Allan[1]*

[1] Faculty of Life Sciences, University of Manchester, Manchester, UK
[2] Laboratory of Molecular Neuroendocrinology, Institute of Experimental Medicine, Budapest, Hungary

Edited by:
Eric W. Roubos, Radboud University Nijmegen, Netherlands

Reviewed by:
Jaleel A. Miyan, The University of Manchester, UK
James Downing, Liverpool John Moores University, UK
Eric W. Roubos, Radboud University Nijmegen, Netherlands

***Correspondence:**
Adam Denes, Laboratory of Molecular Neuroendocrinology, MTA KOKI, Szigony u. 43., 1083 Budapest, Hungary
e-mail: adam.denes@manchester.ac.uk; denesa@koki.hu;
Stuart M. Allan, Faculty of Life Sciences, University of Manchester, AV Hill Building, Oxford Road, Manchester M13 9PT, UK
e-mail: stuart.allan@manchester.ac.uk

Acute brain injury results in peripheral inflammatory changes, although the impact of these processes on neuronal death and neuroinflammation is currently unclear. To facilitate the translation of experimental studies to clinical benefit, it is vital to characterize the mechanisms by which acute brain injury induces peripheral inflammatory changes, and how these are affected by surgical manipulation in experimental models. Here we show that in mice, even mild surgical manipulation of extracranial tissues induced marked granulocyte mobilization (300%) and systemic induction of cytokines. However, intracranial changes induced by craniotomy, or subsequent induction of focal cerebral ischemia were required to induce egress of CXCR2-positive granulocytes from the bone marrow. CXCR2 blockade resulted in reduced mobilization of granulocytes from the bone marrow, caused an unexpected increase in circulating granulocytes, but failed to affect brain injury induced by cerebral ischemia. We also demonstrate that isoflurane anaesthesia interferes with circulating leukocyte responses, which could contribute to the reported vascular and neuroprotective effects of isoflurane. In addition, no immunosuppression develops in the bone marrow after experimental stroke. Thus, experimental models of cerebral ischemia are compromised by surgery and anaesthesia in proportion to the severity of surgical intervention and overall tissue injury. Understanding the inherent confounding effects of surgical manipulation and development of new models of cerebral ischemia with minimal surgical intervention could facilitate better understanding of interactions between inflammation and brain injury.

Keywords: experimental stroke, bone marrow, granulocyte, CXCR2, anaesthesia

INTRODUCTION

Brain injury due to cerebral ischemia, hemorrhage, or head trauma results in early activation of the immune system, followed later by immunosuppression (Dirnagl et al., 2007; Denes et al., 2010b). Activated immune cells and inflammatory mediators contribute to neuroinflammation and overall outcome after experimental brain injury. Blockade of toll-like receptors, proinflammatory mediators such as interleukin-1, and immune cells such as T cells, neutrophils, or mast cells, is protective in experimental models of brain injury (Iadecola and Anrather, 2011; Smith et al., 2013). However, oversuppression of the immune system in response to brain injury gives rise to opportunistic infections leading to impaired recovery and death of patients and experimental animals (Dirnagl et al., 2007; Murray et al., 2013). Therefore, understanding interactions between peripheral inflammatory responses and brain injury could pave the way for novel therapeutic interventions.

Lack of translation in stroke has triggered intense discussions, leading to improved preclinical guidelines on experimental modeling (Fisher et al., 2009). Yet, compared to the large number of clinical studies on peripheral inflammatory changes in stroke, studies on systemic inflammatory mechanisms in experimental animals remain relatively sparse.

We and others have shown that experimental stroke results in the activation of inflammatory responses in various immune organs such as the spleen, blood, or bone marrow (Offner et al., 2006a,b; Chapman et al., 2009; Denes et al., 2011). Using a filament model of transient, focal cerebral ischemia we also showed that anaesthesia and surgical intervention compromise stroke-induced inflammatory responses in the periphery (Denes et al., 2011). However, a systematic analysis of stroke-induced peripheral inflammatory actions, including the assessment of confounding effects of surgical manipulations has not yet been performed in experimental stroke that involves craniotomy. Such investigations could distinguish model-specific and ischemia-induced effects of experimental stroke on immune responses and yield important data for translation to patients.

Thus, we examined the effects of anaesthesia, surgical manipulation, and experimental stroke induced by transient, distal middle cerebral artery occlusion with craniotomy on peripheral inflammatory responses. We show that both isoflurane anaesthesia and surgical intervention induce changes in peripheral

immune cell populations in the absence of stroke, and are likely to contribute to systemic inflammatory responses induced by experimental brain injury. These effects should be accounted for in experimental stroke modeling.

MATERIALS AND METHODS

MICE

Male 8–12 week-old C57BL/6 mice ($n = 58$) were kept at 21 ± 1°C and 65% humidity with a 12 h light-dark cycle and had free access to food and water. All animal procedures were performed under appropriate project license authority and adhered to the UK Animals (Scientific Procedures) Act (1986) and were in accordance with STAIR and ARRIVE (Fisher et al., 2009; Kilkenny et al., 2010) guidelines.

MIDDLE CEREBRAL ARTERY OCCLUSION (MCAo)

Distal, transient focal cerebral ischemia was induced as described earlier (Pradillo et al., 2009, 2012). Briefly, mice were anaesthetized with isoflurane and ischemia was induced by a transient ligature (60 min) of the left MCA trunk with a 10-0 suture (Prolene, Ethicon, Somerville, NJ, USA). Occlusion and reperfusion were confirmed visually under the surgical microscope. Core body temperature was maintained at 37.0 ± 0.5°C throughout the surgery by a heating blanket (Homeothermic Blanket Control Unit; Harvard Apparatus, Kent, UK) and monitored after recovery. After surgery, animals were returned to their cages and allowed free access to water and food. It was decided, *a priori*, to exclude from the study those animals that showed brain hemorrhage at any time of the surgery or with no reperfusion (2 mice). The survival rate was 100%.

SURGICAL CONTROLS

We investigated the effect of surgical manipulation and anaesthesia in the absence of MCAo on cellular and cytokine responses. To achieve this, three separate experimental conditions were used; the first involved only anaesthesia with no surgical manipulation (termed as "isoflurane"), the second exposure of the skull bone but no craniotomy (termed as "sham no cran.") and the third full craniotomy (termed as "sham"). Except for naive animals, all groups of mice were kept under isoflurane anaesthesia for the same time period (75 min for control experimental conditions and in the "MCAo" group).

BLOOD SAMPLING AND TISSUE PROCESSING

Blood samples were taken from the tail vein and from the right cardiac ventricle, using 3.8% sodium citrate 1:10 as an anticoagulant. Repeated tail vein samples were collected at various time points, with equal sampling times across different treatment groups: prior to surgery ("naïve"), 60 min after the onset of ischemia or corresponding control surgery ("0 min reperfusion"), and before transcardial perfusion (at 4 or 72 h reperfusion, not shown). Terminal cardiac blood samples were collected immediately prior to transcardial perfusion from naive mice, and from mice that had undergone surgical interventions, at 4 or 72 h reperfusion. Blood sample data from different vascular beds were analyzed separately. To avoid potential confounding effects of repeated tail vein samples at later time points, cardiac blood samples are presented throughout the manuscript, with the exception of **Figures 1A,B**. To isolate the bone marrow and the spleen, mice were perfused transcardially with saline under isoflurane anaesthesia. Brains were subsequently perfused with 4% paraformaldehyde, post-fixed for 24 h, cryoprotected in 20% sucrose/PBS, and sectioned (20 μm diameter) on a sledge microtome. Organs were homogenized as described previously (Denes et al., 2010a).

CXCR2 BLOCKADE

To prevent CXCR2-mediated release of granulocytes from the bone marrow, mice in the MCAo group were treated intraperitoneally with a selective CXCR2 antagonist (SB225002) or vehicle. SB225002 was dissolved in DMSO (stock), diluted in sterile saline 1:40 and injected as 2 mg/kg, 200 μl/mouse, 20 min prior to induction of anaesthesia for MCAo surgery.

In vitro STIMULATION OF BONE MARROW CELLS

Bone marrow cells were isolated 72 h after 60 min MCAo or the surgery control and stimulated *in vitro* with 1 μg/ml bacterial lipopolysaccharide (LPS, *E. coli* O26:B6), at a density of 7.5×10^6 cells/mL in RPMI medium, supplemented with 10% fetal calf serum and penicillin/streptomycin to investigate cytokine production. After 3 h incubation at 37°C, cells were pelleted with centrifugation at 400 × *g*, the supernatant was collected, and cells were lysed in lysis buffer.

CYTOMETRIC BEAD ARRAY

Circulating levels of IL-6 and KC (CXCL-1), and levels of IL-6 in bone marrow cell culture supernatants and lysates were measured by using CBA Flex Sets (BD Biosciences, UK) according to the manufacturers protocol. The detection limit for each cytokine was 5–10 pg/ml.

FLOW CYTOMETRY

Spleen, bone marrow, and blood cells were isolated and stained with appropriate combinations of CD45-PerCP-Cy5.5, Ly6c-PerCP-Cy5.5, CD4-PE-Cy7, CD8-PE, CD3-APC, CD19-PE-Cy7, MHCII-APC (eBioscience, UK), and Ly6G-PE (1A8, BD Biosciences, UK) following Fc receptor blockade (eBioscience). Contaminating red blood cells in bone marrow and spleen samples were removed by ACK solution, and FACS lysing solution (BD Biosciences) was used to remove red blood cells from blood samples. Total blood cell counts were calculated by using 15 μm polystyrene microbeads (Polysciences, 18328-5). Cells were acquired on an LSRII flow cytometer, using FACS Diva software (BD Biosciences, UK). Except for the 72 h time point, flow cytometric data have been pooled from three independent experiments, in which all experimental groups have been represented by at least one animal. Due to red blood cell contamination and cell labeling artifacts $n = 6$ blood samples, $n = 3$ bone marrow samples and $n = 4$ spleen samples have been excluded from analysis across experiments, *pre-hoc*.

RANDOMIZATION, QUANTIFICATION, AND STATISTICAL ANALYSIS

Animals were randomized for experiments and all quantitative analyses were performed in a blinded manner. Group sizes were determined based on our earlier *in vivo* experiments using

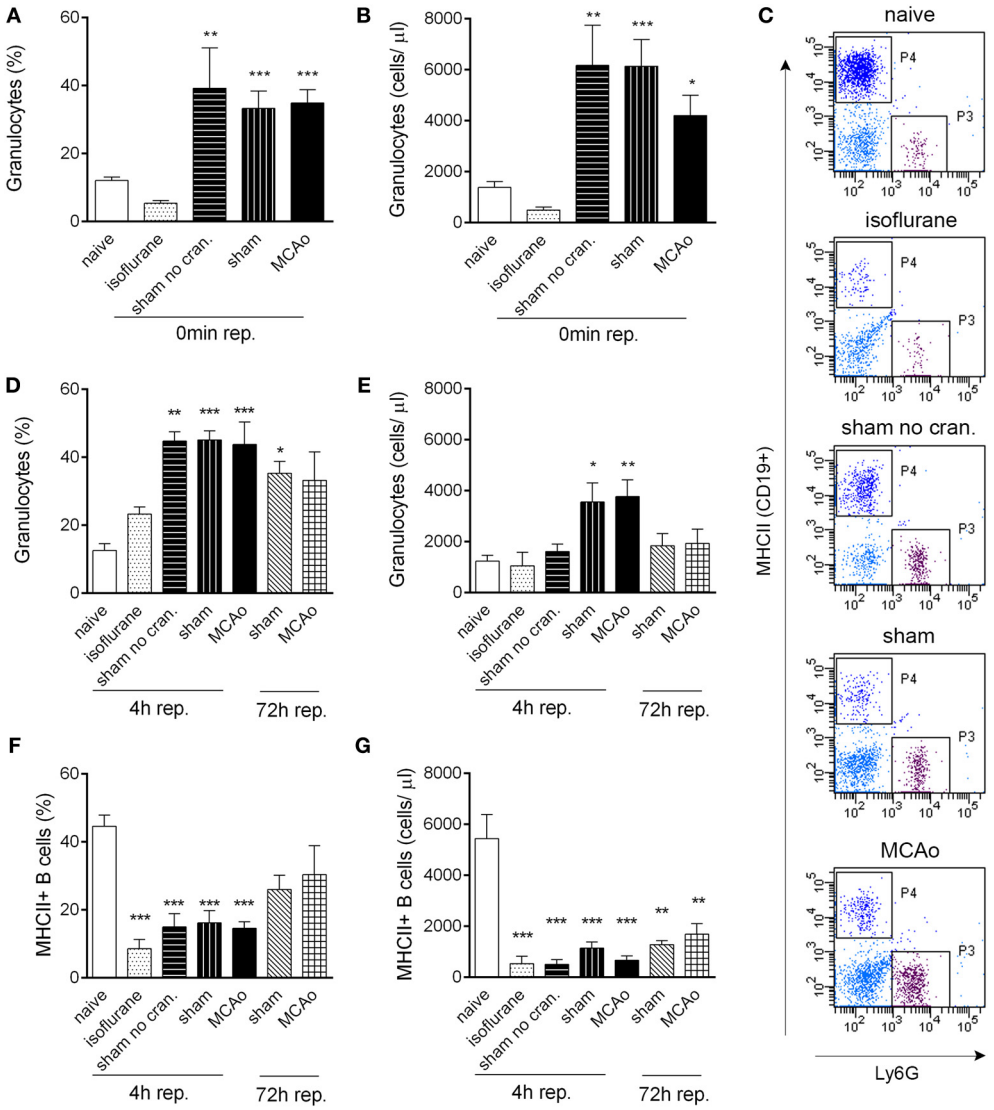


FIGURE 1 | Tissue injury, craniotomy, and anaesthesia result in rapid alteration of leukocyte responses. Extracranial tissue injury results in increased proportion **(A)** and cell numbers **(B)** of granulocytes in the circulation, which is indistinguishable from changes induced by sham surgery or MCAo. Blood samples taken from the tail vein prior to surgery and 60 min after the onset of MCAo/control surgery (0 min reperfusion) are shown. **(C)** Representative dot plots showing terminal cardiac blood samples at 4 h reperfusion for graphs **(D–G)**. P3 gate: Ly6G+ granulocytes, P4 gate: MHCII+ B cells (dark blue color indicates CD19+ positive cells). Proportion **(D)** and total cell numbers **(E)** of granulocytes in cardiac blood samples at 4 and 72 h reperfusion. Proportion **(F)** and total cell numbers **(G)** of MHCII+ B cells (CD19+) in cardiac blood samples at 4 and 72 h reperfusion. Sham no cran.—sham surgery without craniotomy. $n = 5, 4, 4, 5, 6–7, 3, 3$ in naïve, isoflurane, sham no cran., sham, MCAo, sham 72 h, and MCAo 72 h, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. naive. Light blue color indicates negative (unstained) population.

this experimental model. Normality of data sets was assessed using GraphPad Prism (KS normality test). Statistical analysis was performed by One-Way or Two-Way ANOVA followed by Tukey's or Bonferroni's *post-hoc* multiple-comparison, using GraphPad Prism 5 software. In case of non-parametric data, significance was confirmed by non-parametric *t*-test (Mann–Whitney *U*-test) or Kruskal–Wallis test followed by Dunn's multiple comparisons test. All data are expressed as mean ± standard error of the mean (s.e.m). $P < 0.05$ was considered statistically significant.

RESULTS

SURGICAL MANIPULATION AND ANAESTHESIA ALTER LEUKOCYTE RESPONSES IN THE BLOOD

Surgical manipulation of extracranial tissues was sufficient to induce rapid mobilization of granulocytes. In serial blood samples taken from the tail vein, the proportion of granulocytes (Ly6G+ CD11b+ SSC^{high} cells) increased from 12 to 40% within 70–80 min ("0 min reperfusion") in response surgery without craniotomy ("sham no cran.," **Figure 1A**), but not after isoflurane anaesthesia alone, representing a ∼3-fold increase in total

granulocyte numbers (**Figure 1B**). Granulocyte mobilization was not altered further by craniotomy or MCAo (**Figures 1A,B**). Granulocytosis was also evident 5–5.5 h after initiation of surgery in cardiac, terminal blood samples (termed as "4 h reperfusion," **Figures 1C,D**), although total granulocyte numbers were lower compared to the earlier time point and were significantly elevated only in response to craniotomy or MCAo (**Figure 1E**). An increased proportion of granulocytes in the sham and MCAo groups was observed up to 72 h after surgery (**Figure 1D**).

CD19+ MHCII+ B cells decreased profoundly (by 80–90%) in the blood in response to anaesthesia alone, which was apparent in all other groups of mice that underwent surgical manipulation or MCAo, at 4 h reperfusion (**Figures 1F,G**). Similarly, anaesthesia alone resulted in reduced circulating CD4+ and CD8+ T cell numbers within 4 h (**Figures 2A–C**). Interestingly, craniotomy and MCAo appeared to raise T cell numbers over levels after anaesthesia alone, which was more apparent in the case of CD4+ T cells (**Figures 2B,C**). Circulating B cells and CD8+ T cells remained significantly lower in number in surgery control groups and after MCAo compared to naïve mice at 72 h reperfusion (**Figures 1F,G**, **2A,C**).

GRANULOCYTE RELEASE FROM THE BONE MARROW INCREASES PROPORTIONALLY TO THE LEVEL OF SURGICAL STRESS AND BRAIN INJURY

MCAo resulted in mobilization of granulocytes from the bone marrow at 4 h reperfusion (**Figure 3A**). Within the granulocyte population, CXCR2+ granulocytes (Ly6G+ CD11b+ SSC^{high} cells) showed a decrease in the bone marrow proportional to the level of surgical stress, reaching a significant 50% reduction after craniotomy and an over 60% reduction in response to MCAo (**Figure 3B**). Correspondingly, KC (CXCL-1) and IL-6 concentrations in the circulation increased by 20–40-fold after sham surgery and MCAo (**Figures 3C,D**). Circulating KC levels showed a significant ($P < 0.01$) negative correlation with numbers of CXCR2+ granulocytes in the bone marrow at 4 h reperfusion (not shown). Anaesthesia alone appeared to increase B cells and T cells in the bone marrow (**Figures 3E–G**) similarly to our earlier findings (Denes et al., 2011).

BONE MARROW CELLS DO NOT EXHIBIT A SUPPRESSED RESPONSE AFTER CEREBRAL ISCHEMIA

At 72 h reperfusion when systemic immunosuppression is apparent after cerebral ischemia (Prass et al., 2003), bone marrow cells showed no difference in LPS-induced IL-6 production *in vitro* as assessed in cell lysates (**Figure 4A**) and cell culture supernatants (**Figure 4B**), indicating that at this time point cells residing in the bone marrow maintain their ability to respond to endotoxin.

SURGICAL INTERVENTION AND CEREBRAL ISCHEMIA ALTER SPLENIC GRANULOCYTE RESPONSES

Surgical manipulation of extracranial tissues with or without craniotomy resulted in an early recruitment of granulocytes in

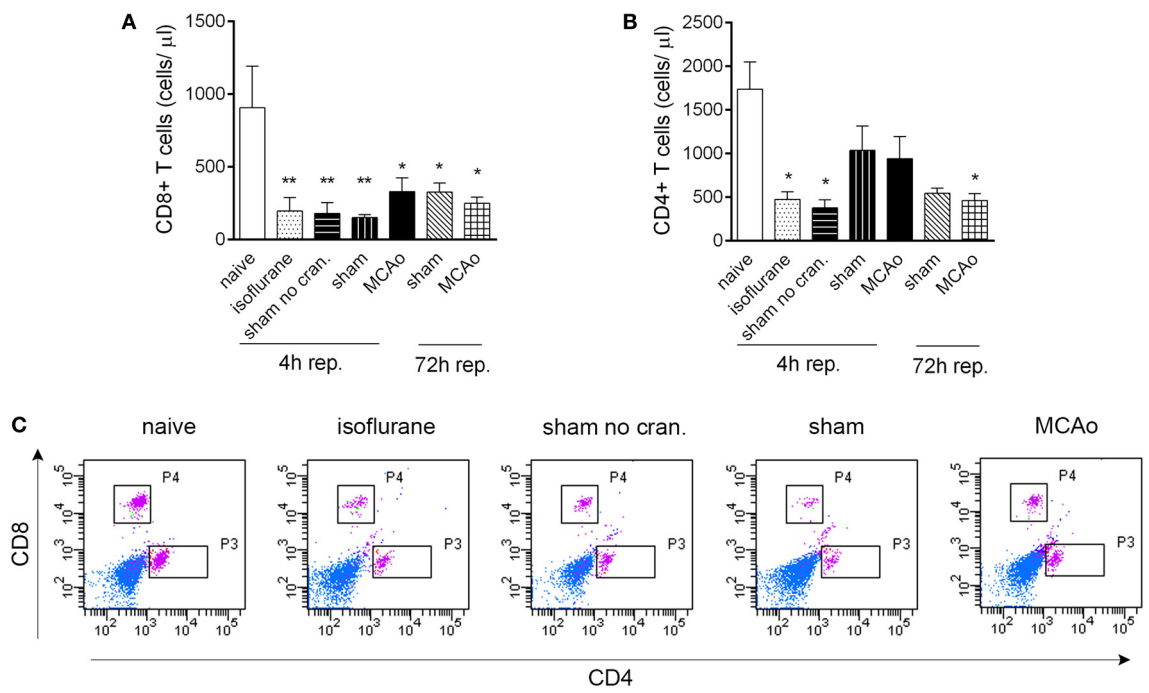


FIGURE 2 | Isoflurane anaesthesia results in reduced circulating T cell numbers. CD8+ T cells **(A)** and CD4+ T cells **(B)** are reduced in number in cardiac blood samples within 4 h following isoflurane anaesthesia. **(C)** Representative dot blots showing CD8+ and CD4+ T cells in cardiac blood samples 4 h after reperfusion, control surgeries, or anaesthesia. Purple color indicates cells gated on CD3. Sham no cran.—sham surgery without craniotomy. $n = 4, 4, 4, 5, 6, 3, 3$ in naïve, isoflurane, sham no cran., sham, MCAo, sham 72 h, and MCAo 72 h, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. naive. Light blue color indicates negative (unstained) population.

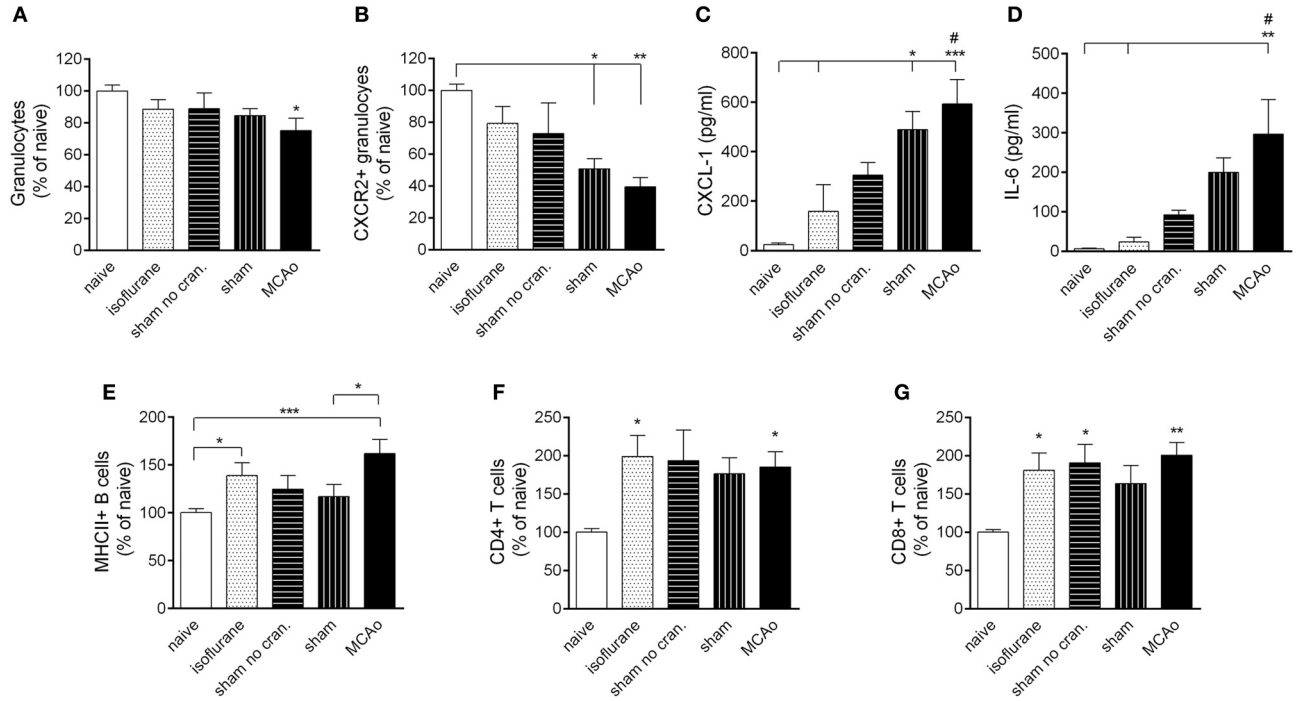


FIGURE 3 | Surgical intervention and anaesthesia result in altered leukocyte responses in the bone marrow. (A) Bone marrow granulocytes (Ly6G+, CD11b+, SSC^{high} cells) are reduced within 4 h after MCAo. **(B)** Both MCAo and craniotomy result in reduced CXCR2+ granulocytes in the bone marrow at 4 h reperfusion. KC (CXCL1, **C**) and IL-6 **(D)** levels increase in the circulation within 4 h proportionally to the amount of surgical stress. Anaesthesia results in increased MHCII+ B cells **(E)** CD4+ T cells **(F)** and CD8+ T cells **(G)** in the bone marrow within 4 h. Sham no cran.—sham surgery without craniotomy. $n = 7$, 5, 6, 7, 7–8 in naïve, isoflurane, sham no cran., sham, and MCAo, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. naïve, unless indicated otherwise on the graphs; $^{\#}P < 0.05$ vs. isoflurane.

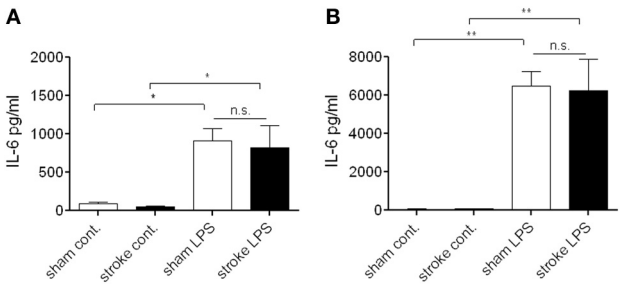


FIGURE 4 | Bone marrow cells do not exhibit a suppressed response to endotoxin after distal tMCAo. Bone marrow cells were isolated 72 h after MCAo and stimulated *in vitro* with bacterial lipopolysaccharide (LPS) to investigate cytokine production. Cell lysates **(A)** and supernatants **(B)** were measured for IL-6 production and release. n.s., not significant. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

the spleen (**Figure 5A**). This effect was not apparent in the CXCR2+ granulocyte population (**Figure 5B**). MCAo significantly reduced splenic total granulocyte- and CXCR2+ granulocyte numbers (**Figures 5A,B**) compared to sham surgery at 4 h reperfusion. No changes were observed in B cell or T cell populations in the spleen in any of the surgical groups (**Figures 5C–E**).

CXCR2 BLOCKADE PREVENTS GRANULOCYTE MOBILIZATION FROM THE BONE MARROW BUT DOES NOT ALTER ISCHEMIC BRAIN INJURY

The selective CXCR2 inhibitor SB225002 (White et al., 1998) prevented granulocyte release from the bone marrow as confirmed by a significant reduction of granulocytes in the bone marrow in vehicle-treated mice 4 h after MCAo compared to mice treated with SB225002 (**Figures 6A,B**). Similarly to our earlier experiments using the intraluminal filament method to induce cerebral ischemia (Denes et al., 2011) bone marrow granulocyte numbers were stabilized by 24 h post MCAo (**Figure 6A**). To our surprise, CXCR2 blockade although reducing granulocyte release from the bone marrow, resulted in an increase in circulating granulocytes at 4 h reperfusion compared to vehicle (**Figure 6C**). We investigated whether CXCR2 blockade resulted in any changes in infarct size, but no difference between SB225002 treatment and vehicle was observed (**Figure 6D**), indicating that CXCR2-mediated signals do not contribute substantially to brain injury in the current experimental model of cerebral ischemia.

DISCUSSION

Here we present evidence that even mild surgical manipulation results in marked systemic granulocyte mobilization responses. Granulocyte mobilization is proportional to the level of surgical stress and is further augmented by brain injury. Granulocyte responses to cranial injury or cerebral ischemia involve primarily

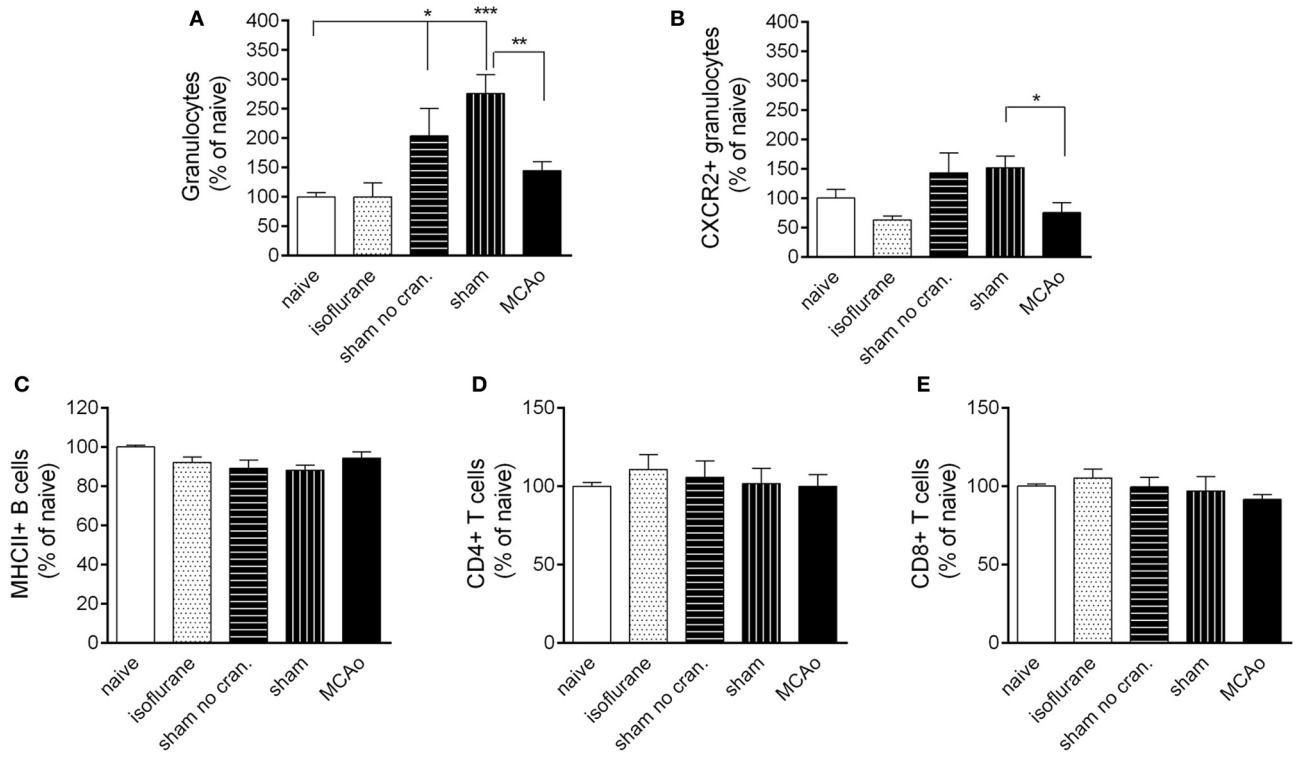


FIGURE 5 | Leukocyte responses in the spleen. Changes in total granulocytes (Ly6G+, CD11b+, SSC^{high} cells, **A**) and CXCR2+ granulocytes **(B)** in the spleen 4 h following reperfusion or control surgical interventions. No changes are observed in splenic MHCII+ B cells **(C)** CD4+ T cells **(D)** and CD8+ T cells **(E)** at 4 h reperfusion. Sham no cran.—sham surgery without craniotomy. $n = 6, 6, 6, 5, 6$ in naïve, isoflurane, sham no cran., sham, and MCAo, respectively. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. naïve; $^{**}P < 0.01$ **(A)** and $^{*}P < 0.05$ **(B)** sham vs. MCAo.

the mobilization of CXCR2-positive granulocytes, which is not seen after manipulation of extracranial tissues. In addition, we show that anaesthesia profoundly alters leukocyte responses, an effect that lasts for several hours or even days. Collectively, these data highlight the role of anaesthesia and surgical intervention in systemic inflammatory responses, which could have an impact on experimental models of cerebral ischemia, but could also serve important information for the management of patients subjected to various surgeries in the clinic.

In our previous study we revealed changes in the bone marrow in response to transient, focal cerebral ischemia induced by an intraluminal filament that is advanced through the internal carotid artery to occlude the MCA (Denes et al., 2011). Although we demonstrated changes induced by brain injury itself, we also showed that anaesthesia and surgical intervention could impact on bone marrow leukocyte responses (Denes et al., 2011). Since the filament MCAo model involves surgical manipulation around the neck including exposure of the salivary glands, the vagus nerve and other tissues around the trachea, in the present study we performed a systematic analysis of bone marrow, spleen, and blood responses, using a distal MCAo model, to reveal stroke-specific changes in leukocyte mobilization as well as the impact of surgical intervention and anaesthesia. Since the present model includes craniotomy, a potential confounder that disturbs the internal milieu of the brain, we also included control surgeries during which only extracranial tissues were manipulated. Extracranial manipulation consisting of an incision on the skin and temporary dislocation of small pericranial muscles was sufficient to generate a systemic inflammatory response evidenced by granulocyte mobilization and a 10-fold increase in circulating KC levels. Since these changes did not involve major alterations in CXCR2+ granulocytes, identification of the mechanisms involved (contribution of bone marrow-derived cells, signals that initiate and maintain systemic granulocyte responses, etc.) warrant further investigation. It is likely that activation of the HPA axis and the autonomic nervous system (Elenkov et al., 2000) could contribute to surgery-induced peripheral changes, however the exact signals mediating leukocyte responses need to be identified. In fact, we confirmed here our earlier findings showing an increased mobilization of CXCR2-positive granulocytes in response to brain injury, compared with other surgical manipulations (Denes et al., 2011).

We found that isoflurane anaesthesia alone resulted in a profound reduction in circulating lymphocytes within hours. Some effects of isoflurane on leukocyte activation have been reported earlier (Yuki et al., 2012; Carbo et al., 2013), and a decrease in Th1/Th2 ratio in the blood has been observed in patients undergoing craniotomy in response to isoflurane (Inada et al., 2004). However, the present study demonstrates a rapid and sustained reduction in circulating T cell numbers induced by isoflurane

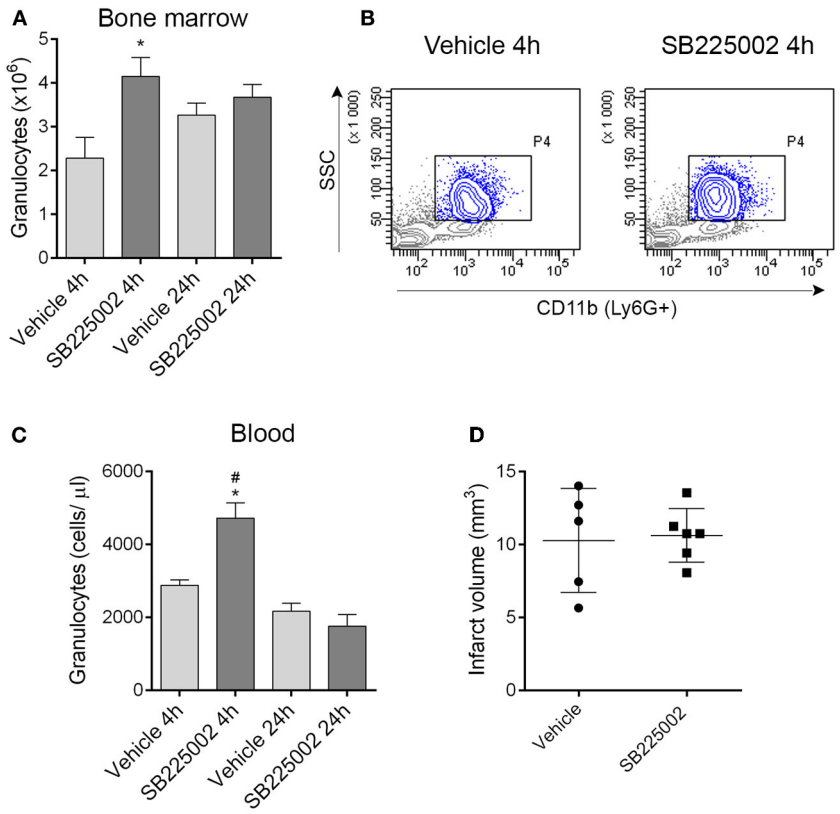


FIGURE 6 | Blockade of CXCR2-mediated granulocyte release from the bone marrow does not alter infarct size after experimental stroke. (A) Treatment with the selective CXCR2 antagonist SB225002 prevents granulocyte release from the bone marrow after MCAo compared to vehicle at 4 h reperfusion. Total granulocyte numbers recovered from 1 femur and 1 tibia are shown. **(B)** Representative density plots showing CD11b+ SSC^{high} granulocytes (gated also on Ly6G+, blue) in the bone marrow at 4 h reperfusion. **(C)** SB225002 increases granulocyte numbers in the cardiac blood at 4 h reperfusion after MCAo. **(D)** Infarct size is not altered in response to SB225002 treatment at 24 h reperfusion, after MCAo. **(A–C)** $n = 3$, 3, 5, 5 in vehicle 4 h, SB225002 4 h, vehicle 24 h, and SB225002 24 h samples, respectively, **(D)** $n = 5$–6. $^{*}P < 0.05$ vs. vehicle 4 h, $^{\#}P < 0.05$ vs. 24 h time points.

anaesthesia, which could have important implications clinically and also in models of experimental stroke. The contribution of T cells to the ischemic brain injury is well documented (Iadecola and Anrather, 2011). Since volatile anaesthetics can exert neuroprotective properties (Kawaguchi et al., 2005), it is possible that at least in part, these actions could be mediated via blunted T cell responses.

The spleen is profoundly affected by brain injury (Offner et al., 2006b), leading to loss of B cells and development of immunosuppression, however, much less is known about how cerebral ischemia contributes to responses of myeloid cells in the spleen. In fact, a population of splenic monocytes are released rapidly upon activation and contributes to ischemic processes in the heart (Swirski et al., 2009; Leuschner et al., 2012). We found that surgical manipulation results in increased granulocyte recruitment to the spleen whilst cerebral ischemia reduced surgery-induced increases in splenic granulocytes. Although the role and mechanisms of surgery-induced splenic granulocytosis requires further investigations, this scenario indicates that the effect of surgical manipulation on leukocyte responses in peripheral organs is likely to be a confounder in current experimental stroke models.

CXCR2 is the best characterized cell surface receptor on granulocytes involved in cell mobilization to stimuli mediated by CXCL1 (KC) and CXCL2/3 (MIP-2) (Matzer et al., 2004; Eash et al., 2010; Veenstra and Ransohoff, 2012). Although brain injury-induced granulocyte mobilization from the bone marrow was prevented by CXCR2 blockade, circulating granulocytes increased in response to SB225002 treatment. CXCR2 inhibition was found effective to reduce IL-8- or KC-induced granulocyte mobilization in the blood and block granulocyte egress from the bone marrow (White et al., 1998; Martin et al., 2003; Eash et al., 2010). We show here that very early granulocyte mobilization responses in response to brain injury are not dependent on CXCR2, and might not (or only in part) require the contribution of the bone marrow in the current experimental model. This could explain the lack of an effect by CXCR2 blockade on the size of ischemic brain injury, which has also been confirmed by another study (Brait et al., 2011). Brain injury in different experimental models might not be influenced by peripheral leukocyte actions to the same extent. For example, blockade of granulocyte responses or hematopoietic MyD88-dependent actions is not associated with reduced central inflammation or brain damage

after cold-induced cortical injury (Koedel et al., 2007). In contrast, neutrophils contribute to brain injury when experimental stroke is preceded by systemic inflammation (McColl et al., 2007). Therefore, the source of neutrophils mobilized after brain injury and their contribution to the population that is recruited into the brain need to be investigated in further studies. Stroke in patients results in increased circulating granulocyte numbers and loss of T cells (Vogelgesang et al., 2008), which corresponds to the findings presented in our study. Surgical interventions and anaesthesia also influence leukocyte responses in patients (Inada et al., 2004). However, similarly to most experimental stroke models, the MCAo model is inherently confounded by effects of anaesthesia and surgical intervention, and we show that surgical interventions alone are capable of inducing profound changes in leukocyte responses. These changes might contribute to and/or alter brain injury in experimental models. Since circulating white blood cell- and blood cytokine data from stroke patients and from those undergoing anaesthesia or surgical interventions are widely available, blood cell responses and mechanisms of brain injury in experimental models should be investigated in a translational context.

In conclusion, our data indicate that anaesthesia- and surgery-induced leukocyte responses interact with those induced by experimental stroke, which needs to be accounted for in experimental models. Revealing the mechanisms of rapid, surgery- or brain injury-induced granulocyte responses could be fundamental for the development of new therapeutic regimen in a wide variety of diseases.

ACKNOWLEDGMENTS

We are grateful for funding provided by the Medical Research Council and European Union's Seventh Framework Program (FP7/2008–2013) under Grant agreements Nos. 201024 and 202213 (European Stroke Network) and TÁMOP-4.2.4.A/2-11/1-2012-0001 (Adam Denes).

REFERENCES

Brait, V. H., Rivera, J., Broughton, B. R., Lee, S., Drummond, G. R., and Sobey, C. G. (2011). Chemokine-related gene expression in the brain following ischemic stroke: no role for CXCR2 in outcome. *Brain Res.* 1372, 169–179. doi: 10.1016/j.brainres.2010.11.087

Carbo, C., Yuki, K., Demers, M., Wagner, D. D., and Shimaoka, M. (2013). Isoflurane inhibits neutrophil recruitment in the cutaneous Arthus reaction model. *J. Anesth.* 27, 261–268. doi: 10.1007/s00540-012-1508-1

Chapman, K. Z., Dale, V. Q., Denes, A., Bennett, G., Rothwell, N. J., Allan, S. M., et al. (2009). A rapid and transient peripheral inflammatory response precedes brain inflammation after experimental stroke. *J. Cereb. Blood Flow Metab.* 29, 1764–1768. doi: 10.1038/jcbfm.2009.113

Denes, A., Humphreys, N., Lane, T. E., Grencis, R., and Rothwell, N. (2010a). Chronic systemic infection exacerbates ischemic brain damage via a CCL5 (regulated on activation, normal T-cell expressed and secreted)-mediated proinflammatory response in mice. *J. Neurosci.* 30, 10086–10095. doi: 10.1523/JNEUROSCI.1227-10.2010

Denes, A., Thornton, P., Rothwell, N. J., and Allan, S. M. (2010b). Inflammation and brain injury: acute cerebral ischaemia, peripheral and central inflammation. *Brain Behav. Immun.* 24, 708–723. doi: 10.1016/j.bbi.2009.09.010

Denes, A., McColl, B. W., Leow-Dyke, S. F., Chapman, K. Z., Humphreys, N. E., Grencis, R. K., et al. (2011). Experimental stroke-induced changes in the bone marrow reveal complex regulation of leukocyte responses. *J. Cereb. Blood Flow Metab.* 31, 1036–1050. doi: 10.1038/jcbfm.2010.198

Dirnagl, U., Klehmet, J., Braun, J. S., Harms, H., Meisel, C., Ziemssen, T., et al. (2007). Stroke-induced immunodepression: experimental evidence and clinical relevance. *Stroke* 38, 770–773. doi: 10.1161/01.STR.0000251441.89665.bc

Eash, K. J., Greenbaum, A. M., Gopalan, P. K., and Link, D. C. (2010). CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J. Clin. Invest.* 120, 2423–2431. doi: 10.1172/JCI41649

Elenkov, I. J., Wilder, R. L., Chrousos, G. P., and Vizi, E. S. (2000). The sympathetic nerve–an integrative interface between two supersystems: the brain and the immune system. *Pharmacol. Rev.* 52, 595–638.

Fisher, M., Feuerstein, G., Howells, D. W., Hurn, P. D., Kent, T. A., Savitz, S. I., et al. (2009). Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke* 40, 2244–2250. doi: 10.1161/STROKEAHA.108.541128

Iadecola, C., and Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nat. Med.* 17, 796–808. doi: 10.1038/nm.2399

Inada, T., Yamanouchi, Y., Jomura, S., Sakamoto, S., Takahashi, M., Kambara, T., et al. (2004). Effect of propofol and isoflurane anaesthesia on the immune response to surgery. *Anaesthesia* 59, 954–959. doi: 10.1111/j.1365-2044.2004.03837.x

Kawaguchi, M., Furuya, H., and Patel, P. M. (2005). Neuroprotective effects of anesthetic agents. *J. Anesth.* 19, 150–156. doi: 10.1007/s00540-005-0305-5

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., and Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* 8:e1000412. doi: 10.1371/journal.pbio.1000412

Koedel, U., Merbt, U. M., Schmidt, C., Angele, B., Popp, B., Wagner, H., et al. (2007). Acute brain injury triggers MyD88-dependent, TLR2/4-independent inflammatory responses. *Am. J. Pathol.* 171, 200–213. doi: 10.2353/ajpath.2007.060821

Leuschner, F., Rauch, P. J., Ueno, T., Gorbatov, R., Marinelli, B., Lee, W. W., et al. (2012). Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *J. Exp. Med.* 209, 123–137. doi: 10.1084/jem.20111009

Martin, C., Burdon, P. C., Bridger, G., Gutierrez-Ramos, J. C., Williams, T. J., and Rankin, S. M. (2003). Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity* 19, 583–593. doi: 10.1016/S1074-7613(03)00263-2

Matzer, S. P., Rodel, F., Strieter, R. M., Rollinghoff, M., and Beuscher, H. U. (2004). Constitutive expression of CXCL2/MIP-2 is restricted to a Gr-1high, CD11b+, CD62Lhigh subset of bone marrow derived granulocytes. *Int. Immunol.* 16, 1675–1683. doi: 10.1093/intimm/dxh169

McColl, B. W., Rothwell, N. J., and Allan, S. M. (2007). Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. *J. Neurosci.* 27, 4403–4412. doi: 10.1523/JNEUROSCI.5376-06.2007

Murray, K. N., Buggey, H. F., Denes, A., and Allan, S. M. (2013). Systemic immune activation shapes stroke outcome. *Mol. Cell. Neurosci.* 53, 14–25. doi: 10.1016/j.mcn.2012.09.004

Offner, H., Subramanian, S., Parker, S. M., Afentoulis, M. E., Vandenbark, A. A., and Hurn, P. D. (2006a). Experimental stroke induces massive, rapid activation of the peripheral immune system. *J. Cereb. Blood Flow Metab.* 26, 654–665. doi: 10.1038/sj.jcbfm.9600217

Offner, H., Subramanian, S., Parker, S. M., Wang, C., Afentoulis, M. E., Lewis, A., et al. (2006b). Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *J. Immunol.* 176, 6523–6531.

Pradillo, J. M., Denes, A., Greenhalgh, A. D., Boutin, H., Drake, C., McColl, B. W., et al. (2012). Delayed administration of interleukin-1 receptor antagonist reduces ischemic brain damage and inflammation in comorbid rats. *J. Cereb. Blood Flow Metab.* 32, 1810–1819. doi: 10.1038/jcbfm.2012.101

Pradillo, J. M., Fernandez-Lopez, D., Garcia-Yebenes, I., Sobrado, M., Hurtado, O., Moro, M. A., et al. (2009). Toll-like receptor 4 is involved in neuroprotection afforded by ischemic preconditioning. *J. Neurochem.* 109, 287–294. doi: 10.1111/j.1471-4159.2009.05972.x

Prass, K., Meisel, C., Hoflich, C., Braun, J., Halle, E., Wolf, T., et al. (2003). Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J. Exp. Med.* 198, 725–736. doi: 10.1084/jem.20021098

Smith, C. J., Lawrence, C. B., Rodriguez-Grande, B., Kovacs, K. J., Pradillo, J. M., and Denes, A. (2013). The immune system in stroke: clinical challenges and their translation to experimental research. *J. Neuroimmune Pharmacol.* 8, 867–887. doi: 10.1007/s11481-013-9469-1

Swirski, F. K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., et al. (2009). Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325, 612–616. doi: 10.1126/science.1175202

Veenstra, M., and Ransohoff, R. M. (2012). Chemokine receptor CXCR2: physiology regulator and neuroinflammation controller? *J. Neuroimmunol.* 246, 1–9. doi: 10.1016/j.jneuroim.2012.02.016

Vogelgesang, A., Grunwald, U., Langner, S., Jack, R., Broker, B. M., Kessler, C., et al. (2008). Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke. *Stroke* 39, 237–241. doi: 10.1161/STROKEAHA.107.493635

White, J. R., Lee, J. M., Young, P. R., Hertzberg, R. P., Jurewicz, A. J., Chaikin, M. A., et al. (1998). Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration. *J. Biol. Chem.* 273, 10095–10098. doi: 10.1074/jbc.273.17.10095

Yuki, K., Bu, W., Xi, J., Sen, M., Shimaoka, M., and Eckenhoff, R. G. (2012). Isoflurane binds and stabilizes a closed conformation of the leukocyte function-associated antigen-1. *FASEB J.* 26, 4408–4417. doi: 10.1096/fj.12-212746

Conflict of Interest Statement: The Reviewer Dr. Jaleel A. Miyan declares that, despite being affiliated to the same institution as the authors, the review process was handled objectively. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 September 2013; accepted: 21 December 2013; published online: 17 January 2014.

*Citation: Denes A, Pradillo JM, Drake C, Buggey H, Rothwell NJ and Allan SM (2014) Surgical manipulation compromises leukocyte mobilization responses and inflammation after experimental cerebral ischemia in mice. Front. Neurosci. **7**:271. doi: 10.3389/fnins.2013.00271*

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in
NEUROSCIENCE

REVIEW ARTICLE
published: 29 October 2013
doi: 10.3389/fnins.2013.00204

The gateway theory: bridging neural and immune interactions in the CNS

Daisuke Kamimura, Moe Yamada, Masaya Harada, Lavannya Sabharwal, Jie Meng, Hidenori Bando, Hideki Ogura, Toru Atsumi, Yasunobu Arima and Masaaki Murakami*

JST-CREST, WPI Immunology Frontier Research Center, Graduate School of Frontier Biosciences, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

Edited by:
Jaleel A. Miyan, The University of Manchester, UK

Reviewed by:
Synthia H. Mellon, University of California, San Francisco, USA
Emmanuel Pinteaux, The University of Manchester, UK

****Correspondence:***
Masaaki Murakami, Center for Advanced Science and Innovation, Building A, 4th floor, Office for University-Industory Collaboration, Room 401, 2-1, Yamada-oka, Suita, Osaka 565-0871, Japan
e-mail: murakami@molonc.med.osaka-u.ac.jp

The central nervous system (CNS) is considered an immune-privileged tissue protected by a specific vessel structure, the blood-brain barrier (BBB). Upon infection or traumatic injury in the CNS, the BBB is breached, and various immune cells are recruited to the affected area. In the case of autoimmune diseases in the CNS like multiple sclerosis (MS), autoreactive T cells against some CNS-specific antigens can theoretically attack neurons throughout the CNS. The affected CNS regions in MS patients can be detected as multiple focal plaques in the cerebrum, thoracic cord, and other regions. Vision problems are often associated with the initial phase of MS, suggesting a disturbance in the optic nerves. These observations raise the possibility that there exist specific signals that direct autoreactive T cells past the BBB and into particular sites of the CNS. Using a mouse model of MS, experimental autoimmune encephalomyelitis (EAE), we recently defined the mechanism of the pathogenesis in which regional neural stimulations modulate the status of the blood vessel endothelium to allow the invasion of autoreactive T cells into specific sites of the CNS via the fifth lumbar cord. This gate for autoreactive T cells can be artificially manipulated by removing gravity forces on the hind legs or by electric pulses to the soleus muscles, quadriceps, and triceps of mice, resulting in an accumulation of autoreactive T cells in the intended regions via the activation of regional neurons. Gating blood vessels by regional neural stimulations, a phenomenon we call the gateway theory, has potential therapeutic value not only in preventing autoimmunity, but also in augmenting the effects of cancer immunotherapies.

Keywords: inflammation, cytokines, autoimmune diseases, inflammatory diseases, multiple sclerosis, gateway theory, neural activation, blood-brain barrier

INTRODUCTION

The immune system is a sophisticated protective mechanism that coordinates many types of bone-marrow derived hematopoietic cells including T cells, B cells, dendritic cells, and macrophages among others that have evolved to combat infectious agents and cancerous cells in the body. Careful regulation is required to control the immune system, because uncontrollable immune reactions, or chronic inflammation, are harmful and considered the pathogenesis of many diseases. It has been long recognized yet not well elucidated that neural activation directly influences the immune system. For example, mentally ill stresses in mice promote tumor progression, while good stresses, or eustress, which are induced by an enriched housing environment with increased space, physical activity, and social interactions, suppress it (Cao et al., 2010; Feng et al., 2012; Hassan et al., 2013). In human patients with autoimmune diseases, it has been suggested that psychological stresses including depression and anxiety are associated with disease progression and relapse (Mitsonis et al., 2009; Srivastava and Boyer, 2010). Whereas systemic modulation of the immune system by the nervous system through the secretion of corticosteroid is relatively well characterized, the regional interplay between these two biological systems is just beginning to be elucidated. Here we discuss the regional neuro-immune interactions during chronic inflammations including our recent findings.

INFLAMMATION UNDERLIES MANY TYPES OF CHRONIC DISEASES

Traditionally, inflammation has been defined as pathophysiological symptoms with dolor, calor, rubor, and tumor (Latin for pain, heat, redness, and swelling, respectively). We now know that these symptoms are the consequences of the local accumulation and activation of immune cells that produce soluble factors such as cytokines and lipid mediators. Therefore, it is not an oversimplification to define inflammation as a biological mechanism that locally recruits various immune cells followed by the disruption of local homeostasis. Inflammation, or the accumulation of immune cells, basically acts as a protective mechanism against infectious agents including viruses and bacteria. In fact, mice genetically lacking T lymphocytes, which secrete various cytokines to orchestrate the immune reactions (CD4+ helper T cells) and exert cytotoxic activities that kill unnecessary or undesirable cells, such as infected cells as well as cancer cells (CD8+ killer T cells), are unable to control pathogen invasion via inflammation induction.

At the same time, inflammation has to resolve immediately after these incidences come to an end, since chronically persistent inflammation has a rather adverse effect. Autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS) are good examples of diseases that involve chronic inflammation. Signs of inflammation (i.e., local accumulation of immune cells followed by dysregulation of local homeostasis) are evident in the joints and the central nervous system (CNS) of RA and MS patients, respectively. We have also found the similar phenomena in the target organs of animal models for these diseases. In addition, accumulating evidence indicates that chronic inflammation underlies the pathogenesis of many other types of disorders including neurodegenerative diseases, metabolic syndromes, and even psychiatric diseases (Hamdani et al., 2012; Tabas and Glass, 2013). Thus, understanding the molecular mechanisms that convert protective acute inflammation into harmful chronic inflammation and how chronic inflammation persists is essential to the development of novel therapeutic interventions for many diseases and disorders.

THE INFLAMMATION AMPLIFIER PROVIDES A MOLECULAR MECHANISM FOR CHRONIC INFLAMMATION

ESTABLISHMENT OF A RHEUMATOID ARTHRITIS MODEL, F759 MICE

Interleukin (IL)-6 is a well-studied cytokine that promotes inflammation. In fact, IL-6 is found upregulated in the serum and affected areas of human diseases and disorders such as synovial fluids in the case of RA, while genome-wide association studies (GWAS) have revealed that the IL-6 gene is genetically associated with a wide range of diseases including autoimmunity, neurodegenerative diseases, and cancer (http://geneticassociationdb.nih.gov/). Therefore, the establishment of an IL-6-dependent disease model has been desired for understanding the pathogenesis of various inflammatory diseases. IL-6 signals emanating from IL-6 receptor complexes, gp130 and IL-6R, involve the activation of STAT3, the central transcription factor for the many biological effects of IL-6. SOCS3 is a target gene of IL-6 and known to negatively regulate the signal transduction of IL-6 by binding to the receptor subunit gp130 and Jak kinases (Kamimura et al., 2003; Murakami et al., 2004). Given that IL-6 is abundant in patients with inflammatory disorders, we hypothesized that persistent activation of IL-6/gp130 signaling might induce inflammatory diseases. We therefore generated a knock-in mouse with a point mutation at the binding site of SOCS3 in gp130, thereby enhancing the IL-6 signal pathway (Ohtani et al., 2000). As expected, this mouse strain, called F759 mice, develops RA-like joint diseases spontaneously (Atsumi et al., 2002).

DISCOVERY OF THE INFLAMMATION AMPLIFIER

Breeding experiments with various deficient mouse strains showed the joint disease in F759 mice mainly relies on CD4+ T cells, IL-6, and IL-17, which is a recently reported helper T-cell-derived cytokine involved in autoimmunity and activation of the transcription factor NF-κB (Sawa et al., 2006; Ogura et al., 2008). Indeed, the number of an IL-17-secreting CD4+ T cell subtype (Th17 cells) and the serum levels of IL-17 were increased in F759 mice. Furthermore, systemic expressions of IL-17 or IL-6 in F759 mice accelerated the disease onset and, interestingly, induced the production of IL-6 itself, suggesting a positive feedback loop (Ogura et al., 2008). Moreover, IL-17 expression in F759 mice induced an excessive level of IL-6 and chemokines. *In vitro* experiments using fibroblasts confirmed that IL-6 is a target of the simultaneous treatment of IL-17 and IL-6, but not of either cytokine alone (Ogura et al., 2008). DNA microarray analysis revealed that many chemokines are also targets of this synergism. Since chemokines recruit immune cells to promote inflammation via the dysregulation of local homeostasis, we named this synergistic mechanism the inflammation amplifier (formerly, IL-6 amplifier) (**Figure 1**) (Murakami and Hirano, 2012). Activation of the inflammation amplifier in endothelial cells is induced by the simultaneous activation of NF-κB and STAT3 followed by chemokine expressions. We expected that NF-κB and STAT3 stimulants, whether they are pro-inflammatory or anti-inflammatory cytokines, should enhance the expression of the chemokines. However, anti-inflammatory cytokines might indirectly suppress the activation of the inflammation amplifier by inhibiting activated helper T cells, which express various NF-κB and STAT3 stimulants including IL-17, IL-6, and TNFα. Moreover, as discussed in detail below, the inflammation amplifier, which can be regulated by neural inputs such as norepinephrine (NE), enhances the production of chemokines via NF-κB activation (**Figure 1**) (Arima et al., 2012).

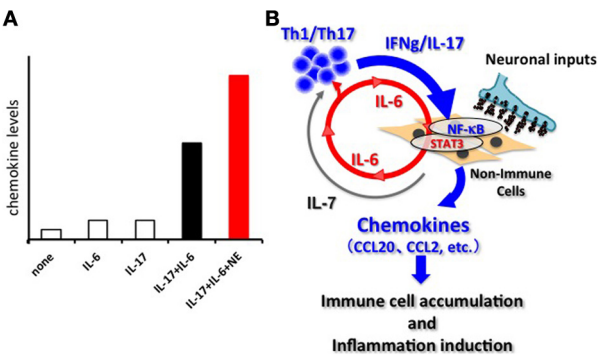


FIGURE 1 | The inflammation amplifier. (A) Image of the activation of the inflammation amplifier in the presence and absence of NE. A combination of IL-17 and IL-6 causes a synergistic effect on the induction of inflammation-amplifier target chemokines such as CCL20 in non-immune cells including fibroblasts, endothelial cells, and certain synovial cells. NE further enhances the chemokine production. Other neurotransmitters can also influence activation of the inflammation amplifier. See Arima et al. (2012) for the original data. **(B)** The synergistic effect seen in **(A)** requires the concomitant activation of two transcriptions factors, NF-kB and STAT3. In the case of autoimmune diseases, Th-17 derived IL-17 induces an initially low amount of IL-6 from non-immune cells via NF-κB activation. Secreted IL-6 together with IL-17 forms a positive feedback loop to produce excessive expression of the target genes including many inflammatory chemokines such as CCL20, CCL2, etc. Consequently, various immune cells are recruited at the region around the target cells via chemokine expression, and inflammation takes place. Persistent activation of the inflammation amplifier, as is the case in F759 mice, drives chronic inflammation. IL-7, a target of the amplifier and derived from non-immune cells, fuels the proliferation, and survival of Th17 cells. The activation of the inflammation amplifier can be modulated by regional neural activation.

THE INFLAMMATION AMPLIFIER IS ASSOCIATED WITH VARIOUS HUMAN DISEASES AND DISORDERS

Mice deficient in gp130 or STAT3 in fibroblasts and/or endothelial cells (i.e., inflammation amplifier-defective non-immune cells) are highly resistant to animal disease models including RA, MS, and allogeneic graft rejection, demonstrating that the inflammation amplifier is critical for multiple chronic inflammatory diseases *in vivo* (Ogura et al., 2008; Murakami et al., 2011; Arima et al., 2012; Lee et al., 2012). To explore the molecular mechanisms underlying the inflammation amplifier, we recently conducted genome-wide functional screening and identified around 1700 candidates that positively regulate it.

Interestingly, genes associated with human diseases including not only autoimmunity, but also metabolic syndromes, neurodegenerative diseases, and other inflammatory diseases including allergies and atopic dermatitis were significantly enriched in the candidate genes (Murakami et al., 2013). Based on these findings, we propose that de novo mutations, expression alterations, or epigenetic changes of these genes in non-immune cells during acute inflammation can cause dysregulation of the amplifier like that observed in F759 mice (i.e., a point-mutation of gp130), turning acute inflammation into a pathogenic chronic version.

NEURO-IMMUNE INTERACTIONS MEDIATE IMMUNE HOMEOSTASIS AND PATHOGENESIS

AUTOREACTIVE CD4+ T CELLS ENTER THE CNS FROM THE FIFTH LUMBAR CORD

MS is a chronic inflammatory disease in the CNS that, according to a recent genome-wide association study, involves CD4+ T cells (International Multiple Sclerosis Genetics et al., 2011). An animal model of MS, experimental autoimmune encephalomyelitis (EAE), is widely used to study the pathogenesis of MS, and autoreactive CD4+ T cells, in particular Th17 cells, have been demonstrated to be essential for the disease induction (Komiyama et al., 2006). In these experiments, mice or rats were immunized with CNS antigens such as myelin oligodendrocyte glycoprotein (MOG) or a proteolipid protein, resulting in the generation of autoreactive CD4+ T cells that target the CNS and the development of MS-like symptoms including progressive paralysis, particularly in the lower body. EAE can also be induced by a passive transfer method that is performed via the intravenous transfer of these autoreactive CD4+ T cells into naïve recipient animals. Indeed, the passive transfer method is suitable to track the behavior of the disease-causing CD4+ T cells without affecting the immune system systemically by adjuvant treatment. We have shown using the transfer method-induced EAE that the inflammation amplifier is critically involved in EAE development, because the disease symptoms were significantly ameliorated in amplifier-deficient mice such as type-1 collagen cre/gp130flox mice (Ogura et al., 2008; Arima et al., 2012). These results challenge traditional theories, because the blood-brain barrier (BBB) should block the migration of the pathogenic CD4+ T cells into the CNS. We thus sought the initial site where autoreactive CD4+ T cells invade the CNS during the development of EAE. A clinical symptom of typical EAE constantly begins with a loss of tonicity in the tail tip, suggesting immune attacks at a particular site in the lower body despite the uniform presence of the autoantigens in the CNS. Whole-mount sections of adult mice at a preclinical stage of EAE revealed that MOG-reactive CD4+ T cells preferentially entered the CNS from the dorsal vessels of the fifth lumbar (L5) cord (Arima et al., 2012).

DORSAL VESSELS OF THE L5 CORD EXPRESS VARIOUS CHEMOKINES VIA ACTIVATION OF THE INFLAMMATION AMPLIFIER, EVEN AT STEADY STATE

This accumulation of CD4+ T cells is dependent on the inflammation amplifier, as expected. In addition, the treatment of mice with antibodies against CCL20, a chemokine potently recruiting Th17 and a target gene of the inflammation amplifier, suppressed the CD4+ T cell accumulation at the L5 dorsal vessels, suggesting that amplifier-mediated CCL20 overproduction causes the recruitment of pathogenic Th17 at the L5 cord (Arima et al., 2012). In fact, not only CCL20, but also various chemokines were upregulated at L5 dorsal vessels during EAE as compared to dorsal vessels from the other spinal levels. Unexpectedly, the upregulation of chemokines at L5 vessels was observed even under steady state (i.e., the natural condition). Given that a certain number of immune cells are present in the CNS, probably for the purpose of immune surveillance, and that the CNS-resident microglia is of bone-marrow origin, the increase of chemokines at the L5 cord may function as a gate for their precursors from the periphery to the CNS, although further studies are needed to confirm this conclusion.

REGIONAL NEURAL ACTIVATION INDUCED BY GRAVITY PLAYS A ROLE IN CHEMOKINE EXPRESSION AT THE L5 GATE

Why the L5 is the location of the gate was answered by investigating a neuro-immune interaction. It is known that sensory neurons in the dorsal root ganglion (DRG) beside the L5 cord are connected to the soleus muscles, the main anti-gravity muscles (Ohira et al., 2004). We considered whether constant stimulation of the soleus muscles by a gravity force might mediate entry of the MOG-specific Th17 cells at the L5 cord. Indeed, when mice were tail-suspended so that the hind limbs were released from the gravity stimuli (**Figure 2**), MOG-specific Th17 cells no longer accumulated at the L5 cord. Instead, these Th17

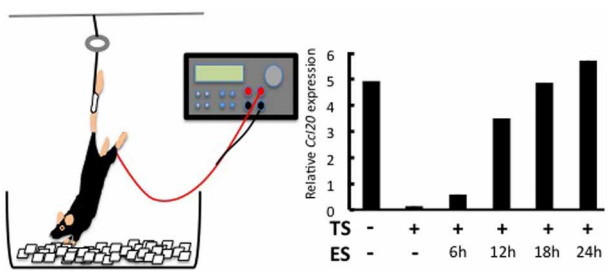


FIGURE 2 | Effects of regional neural activation on the invasion of Th17 cells into the CNS. Tail suspension and electric stimulation of the soleus muscles **(left)**. Tail suspension (TS) reduces CCL20 expression in the L5 dorsal vessel. Electric stimulations (ES) to the soleus muscles of TS mice restore the chemokine expression in a time-dependent manner **(right)**. See Arima et al. (2012) for the original data.

cells accumulated at cervical cords as if a new gate was opened by a gravitational burden on the fore limb muscles (Arima et al., 2012). Consistently, the tail suspension suppressed CCL20 mRNA expression in the L5 dorsal blood vessels and decreased the expression of the neural activation marker c-fos in the L5 DRG. Moreover, artificial electric stimulations to the soleus muscles of the tail-suspended mice restored CCL20 expression (**Figure 2**) and Th17 accumulation at the L5 cord, as well as c-fos levels in the L5 DRG (Arima et al., 2012). These results strongly suggest that neural inputs from the soleus muscles in response to the gravity force play a role in activating the inflammation amplifier and lead to the expression of various chemokines including Th17-attracting CCL20 in L5 dorsal blood vessels.

A SENSORY-SYMPATHETIC INTERACTION IS CRITICAL FOR THE L5 ENTRY OF THE AUTOREACTIVE T CELLS

What mechanisms do afferent sensory neurons from the soleus muscles use to regulate the status of blood vessels at L5? Although a precise neural network remains elusive, we have shown sympathetic nerves to be involved. Blood flow speeds at L5 dorsal vessels become slower when mice are tail suspended, while electronic stimulation of the soleus muscles increases the flow speed. These results clearly suggest that autonomic nerves including sympathetic neurons are involved in the response to the tail suspension. In addition, blood flow speeds in blood vessels other than the L5 region, such as the femoral vessels, brain surface vessels, and the portal vein, are not affected by tail suspension (Arima et al., 2012). Furthermore, treatment with atenolol, a β1 adrenergic receptor antagonist, or prazosin, an α1 adrenergic receptor antagonist, significantly inhibits CCL20 mRNA expression, NF-κB mRNA expression, and MOG-reactive Th17 accumulation at L5 vessels and also suppresses clinical signs of EAE (Arima et al., 2012). Consistent with these *in vivo* results, the addition of NE, which is a neurotransmitter from sympathetic neurons, to a culture of endothelial cells enhances chemokine expressions mediated by the activation of the inflammation amplifier (**Figure 1A**). Thus, anti-gravity responses of the soleus muscles lead to sympathetic nerve stimulation, creating a gateway for immune cells to pass through the CNS via L5 dorsal vessels (Arima et al., 2012). Based on these findings, we concluded that MOG-reactive, disease-causing Th17 cells make use of the L5 gateway to infiltrate the CNS and induce local inflammation by producing cytokines like IL-17 and IL-6, which further induces chemokines via the inflammation amplifier in parenchymal non-immune cells and results in chronic inflammation in the CNS (**Figure 3**). Thus, the neural response triggered by the gravity force creates a gate for immune cells to invade the CNS, although we also hypothesize that the clearance and/or apoptosis of immune cells in the CNS may contribute to the feed-forward mechanism of continued neuroinflammation (Goldmann et al., 2006). Our findings also imply that various types of neural activity affect the disease status differently. Indeed, we found and are currently studying the mechanism for how certain physical or mental stresses cause the deterioration or relapse of EAE in mice, which will shed new light on the molecular mechanism of the relapse and remission of MS.

THE GATEWAY THEORY

Neuronal innervation occurs throughout the body and appropriately controls all organs. One potential strategy to therapeutically manage neuro-immune interactions is to find and stimulate the appropriate peripheral neurons connected to the organs to be treated. To test this theory, we selectively stimulated sensory neurons via electric stimulations of different muscles and observed local chemokine expressions in the vessels of spinal cords as a

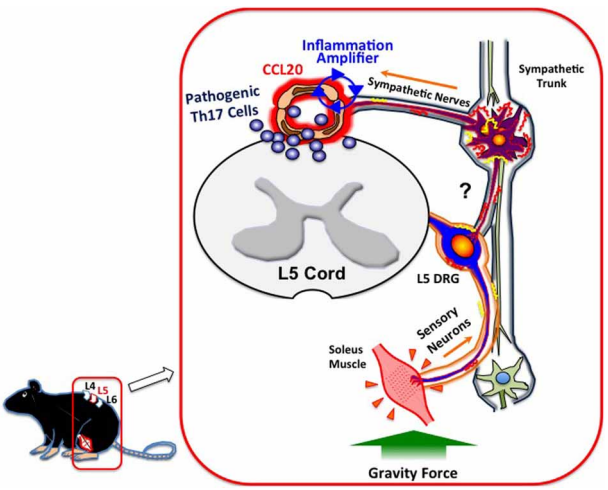


FIGURE 3 | Gateway for autoreactive T cells into the CNS. Continuous stimulation of the soleus muscles by gravity leads to the constant activation of L5 DRG sensory neurons, followed by the activation of sympathetic neurons at the L5 region via unidentified circuits (?). NE, a neurotransmitter from sympathetic neurons, augments the inflammation amplifier at the dorsal vessels of L5, causing an elevation of CCL20 and subsequent Th17 infiltration into the CNS.

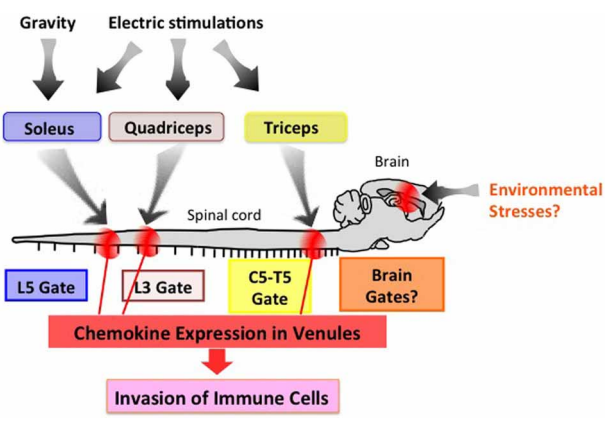


FIGURE 4 | The gateway theory. Electric pulses to the upper arm, thigh, and soleus muscles induce activation of the inflammation amplifier (chemokine upregulation), thereby opening a gate in the dorsal vessels of the fifth cervical to fifth thoracic (C5-T5), third lumbar (L3), and fifth lumbar (L5) cords, respectively. Environmental stresses such as those from social interactions and mental stresses might create a specific gate in the brain. This effect is potentially an attractive target for manipulating immune cell migration *in vivo*.

marker of the inflammatory response. Thigh muscles including the quadriceps are known to be regulated by L3 DRG neurons. Interestingly, electronic stimulation of these muscles followed by L3 DRG activation led to an increased expression of CCL20 in the L3 cord dorsal vessels of mice (Arima et al., 2012). In a similar fashion, chemokine levels in the fifth cervical to fifth thoracic cord vessels were upregulated by stimulations of the epitrochlearis/triceps brachii and upper arm muscles, are controlled by neurons located at the corresponding areas (**Figure 4**) (Arima et al., 2012). Based on these results, we propose "the gateway theory," which describes blood vessels that act as gates for immune cell infiltration into organs and is dependent on regional neural stimulations. The theory is supported by empirical evidence that shows stresses from environments can affect the disease status and trigger relapse in MS patients. Experimentally, we have found that certain environmental stresses influence the disease progression in EAE mice by creating gates for autoreactive helper T cells to enter the CNS (manuscript in preparation). We hypothesize that these stresses create gates at certain CNS vessels via neural control of the inflammation amplifier to modify the disease status at specific vessels (**Figure 4**).

FUTURE DIRECTIONS

Investigations on whether the gateway theory can be more generally applied to tissues beyond the CNS are currently ongoing. If true, artificial manipulation of the gate should have significant clinical benefits, as closing it in normal cells would dampen autoimmune inflammation in a target organ without any systemic immune suppression, while opening it in tumors could enhance cancer immunotherapy. The precise molecular mechanisms for gating still need to be studied, however, to confirm this possibility.

REFERENCES

Arima, Y., Harada, M., Kamimura, D., Park, J. H., Kawano, F., Yull, F. E., et al. (2012). Regional neural activation defines a gateway for autoreactive T cells to cross the blood-brain barrier. *Cell* 148, 447–457. doi: 10.1016/j.cell.2012.01.022

Atsumi, T., Ishihara, K., Kamimura, D., Ikushima, H., Ohtani, T., Hirota, S., et al. (2002). A point mutation of Tyr-759 in interleukin 6 family cytokine receptor subunit gp130 causes autoimmune arthritis. *J. Exp. Med.* 196, 979–990. doi: 10.1084/jem.20020619

Cao, L., Liu, X., Lin, E. J., Wang, C., Choi, E. Y., Riban, V., et al. (2010). Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition. *Cell* 142, 52–64. doi: 10.1016/j.cell.2010.05.029

Feng, Z., Liu, L., Zhang, C., Zheng, T., Wang, J., Lin, M., et al. (2012). Chronic restraint stress attenuates p53 function and promotes tumorigenesis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7013–7018. doi: 10.1073/pnas.1203930109

Goldmann, J., Kwidzinski, E., Brandt, C., Mahlo, J., Richter, D., and Bechmann, I. (2006). T cells traffic from brain to cervical lymph nodes via the cribroid plate and the nasal mucosa. *J. Leukoc. Biol.* 80, 797–801. doi: 10.1189/jlb.0306176

Hamdani, N., Tamouza, R., and Leboyer, M. (2012). Immuno- inflammatory markers of bipolar disorder: a review of evidence. *Front. Biosci.* 4, 2170–2182. doi: 10.2741/534

Hassan, S., Karpova, Y., Baiz, D., Yancey, D., Pullikuth, A., Flores, A., et al. (2013). Behavioral stress accelerates prostate cancer development in mice. *J. Clin. Invest.* 123, 874–886. doi: 10.1172/JCI63324

International Multiple Sclerosis Genetics, C., Wellcome Trust Case Control, C., Sawcer, S., Hellenthal, G., Pirinen, M., Spencer, C. C., et al. (2011). Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 476, 214–219. doi: 10.1038/nature10251

Kamimura, D., Ishihara, K., and Hirano, T. (2003). IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev. Physiol. Biochem. Pharmacol.* 149, 1–38. doi: 10.1007/s10254-003-0012-2

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., et al. (2006). IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* 177, 566–573.

Lee, J., Nakagiri, T., Oto, T., Harada, M., Morii, E., Shintani, Y., et al. (2012). IL-6 amplifier, NF-kappaB-triggered positive feedback for IL-6 signaling, in grafts is involved in allogeneic rejection responses. *J. Immunol.* 189, 1928–1936. doi: 10.4049/jimmunol.1103613

Mitsonis, C. I., Potagas, C., Zervas, I., and Sfagos, K. (2009). The effects of stressful life events on the course of multiple sclerosis: a review. *Int. J. Neurosci.* 119, 315–335. doi: 10.1080/00207450802480192

Murakami, M., Harada, M., Kamimura, D., Ogura, H., Okuyama, Y., Kumai, N., et al. (2013). Disease-association analysis of the IL-6-amplifier, an inflammation-related feedback loop. *Cell Rep*. 3, 946–959. doi: 10.1016/j.celrep.2013.01.028

Murakami, M., and Hirano, T. (2012). The pathological and physiological roles of IL-6 amplifier activation. *Int. J. Biol. Sci.* 8, 1267–1280. doi: 10.7150/ijbs.4828

Murakami, M., Kamimura, D., and Hirano, T. (2004). New IL-6 (gp130) family cytokine members, CLC/NNT1/BSF3 and IL-27. *Growth Factors* 22, 75–77. doi: 10.1080/08977190410001715181

Murakami, M., Okuyama, Y., Ogura, H., Asano, S., Arima, Y., Tsuruoka, M., et al. (2011). Local microbleeding facilitates IL-6- and IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells. *J. Exp. Med.* 208, 103–114. doi: 10.1084/jem.20100900

Ogura, H., Murakami, M., Okuyama, Y., Tsuruoka, M., Kitabayashi, C., Kanamoto, M., et al. (2008). Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. *Immunity* 29, 628–636. doi: 10.1016/j.immuni.2008.07.018

Ohira, Y., Kawano, F., Stevens, J. L., Wang, X. D., and Ishihara, A. (2004). Load-dependent regulation of neuromuscular system. *J. Gravit. Physiol.* 11, P127–P128.

Ohtani, T., Ishihara, K., Atsumi, T., Nishida, K., Kaneko, Y., Miyata, T., et al. (2000). Dissection of signaling cascades through gp130 *in vivo*: reciprocal roles for STAT3- and SHP2-mediated signals in immune responses. *Immunity* 12, 95–105. doi: 10.1016/S1074-7613(00)80162-4

Sawa, S., Kamimura, D., Jin, G. H., Morikawa, H., Kamon, H., Nishihara, M., et al. (2006). Autoimmune arthritis associated with mutated interleukin (IL)-6 receptor gp130 is driven by STAT3/IL-7-dependent homeostatic proliferation of CD4+ T cells. *J. Exp. Med.* 203, 1459–1470. doi: 10.1084/jem.20052187

Srivastava, S., and Boyer, J. L. (2010). Psychological stress is associated with relapse in type 1 autoimmune hepatitis. *Liver Int.* 30, 1439–1447. doi: 10.1111/j.1478-3231.2010.02333.x

Tabas, I., and Glass, C. K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* 339, 166–172. doi: 10.1126/science.1230720

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 July 2013; accepted: 13 October 2013; published online: 29 October 2013. Citation: Kamimura D, Yamada M, Harada M, Sabharwal L, Meng J, Bando H, Ogura H, Atsumi T, Arima Y and Murakami M (2013) The gateway theory: bridging neural and immune interactions in the CNS. Front. Neurosci. 7:204. doi: 10.3389/fnins.2013.00204

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in
NEUROSCIENCE

REVIEW ARTICLE
published: 16 October 2013
doi: 10.3389/fnins.2013.00187

Programming of neuroendocrine self in the thymus and its defect in the development of neuroendocrine autoimmunity

Vincent Geenen[1]*, Gwennaëlle Bodart[1], Séverine Henry[1], Hélène Michaux[1], Olivier Dardenne[1], Chantal Charlet-Renard[1], Henri Martens[1] and Didier Hober[2]

[1] *Department of Biomedical and Preclinical Sciences, Center of Immunoendocrinology, GIGA Research Institute, Fund of Scientific Research, University of Liege, Liege-Sart Tilman, Belgium*
[2] *Laboratory of Virology EA3610, University of Lille 2 and CHRU Lille, Lille, France*

Edited by:
Jaleel A. Miyan, The University of Manchester, UK

Reviewed by:
Francisco Gaytán, University of Cordoba, Spain
Saheem Ahmad, Loyola University Medical Center, Loyola University of Chicago, USA

***Correspondence:**
Vincent Geenen, Center of Immunology, GIGA I3 Research, CHU-B34, B-4000 Liege-Sart Tilman, Belgium
e-mail: vgeenen@ulg.ac.be

For centuries after its first description by Galen, the thymus was considered as only a vestigial endocrine organ until the discovery in 1961 by Jacques FAP Miller of its essential role in the development of T (thymo-dependent) lymphocytes. A unique thymus first appeared in cartilaginous fishes some 500 million years ago, at the same time or shortly after the emergence of the adaptive (acquired) immune system. The thymus may be compared to a small brain or a computer highly specialized in the orchestration of central immunological self-tolerance. This was a necessity for the survival of species, given the potent evolutionary pressure imposed by the high risk of autotoxicity inherent in the stochastic generation of the diversity of immune cell receptors that characterize the adaptive immune response. A new paradigm of "neuroendocrine self-peptides" has been proposed, together with the definition of "neuroendocrine self." Neuroendocrine self-peptides are secreted by thymic epithelial cells (TECs) not according to the classic model of neuroendocrine signaling, but are processed for presentation by, or in association with, the thymic major histocompatibility complex (MHC) proteins. The autoimmune regulator (AIRE) gene/protein controls the transcription of neuroendocrine genes in TECs. The presentation of self-peptides in the thymus is responsible for the clonal deletion of self-reactive T cells, which emerge during the random recombination of gene segments that encode variable parts of the T cell receptor for the antigen (TCR). At the same time, self-antigen presentation in the thymus generates regulatory T (Treg) cells that can inhibit, in the periphery, those self-reactive T cells that escaped negative selection in the thymus. Several arguments indicate that the origin of autoimmunity directed against neuroendocrine glands results primarily from a defect in the intrathymic programming of self-tolerance to neuroendocrine functions. This defect may be genetic or acquired, for example during an enteroviral infection. This novel knowledge of normal and pathologic functions of the thymus constitutes a solid basis for the development of a novel type of tolerogenic/negative self-vaccination against type 1 diabetes (T1D).

Keywords: thymus, self-tolerance, autoimmunity, type 1 diabetes, neuroendocrine self-peptides, oxytocin, insulin-like growth factor 2, AIRE

INTRODUCTION

Galen (129–210 AC), who, with Hippocrates, is recognized as one of the fathers of Western medicine, first observed, behind the sternum and before the cardiac area, an organ that he named "thymus" because of its resemblance to the leaf of the thyme plant *Thymus vulgaris*. Galen also suspected that the thymus was the seat of courage and affection because of its close vicinity to the heart; in Ancient Greece, the word "thumos or thymos (θυμός)" indicated a physical association between breath and blood, and referred to one of Plato's three constituent parts of human psyche.

The function of the thymus remained unknown for many centuries and it was considered only as a vestigial organ that had become redundant both during phylogeny and ontogeny after puberty. Then, at the beginning of the 20th century, Hammar (Sweden) published a series of important studies describing hyperplasia of the thymus in various endocrine diseases including acromegaly and Graves' disease, and after castration (Hammar, 1921). A few years later, Hans Selye reported that massive thymus atrophy is observed soon after administration of glucocorticoids (Selye, 1941), and this dramatic decrease in thymus size soon became a hallmark of the general adaptation and stress syndrome. At that time, the thymus was thought to be an endocrine gland but, despite the identification of several thymic "hormones," the model of endocrine signaling failed to characterize the molecular dialogue between thymic epithelial cells (TECs) and immature thymic T cells (thymocytes). The hypothesis that the thymus had

an endocrine nature then progressively faded, after Miller demonstrated the immunological function of the thymus (Miller, 1961). In this seminal paper, Miller postulated that lymphocytes leaving the thymus are specially selected in an epithelial environment. To the best of our knowledge, Burnet and Mackay published in *The Lancet* the first hypothesis connecting autoimmune diseases with histological abnormalities in the lympho-epithelial structure of the thymus (Burnet and Mackay, 1963). Immunological tolerance was first shown to be induced by thymic epithelial grafts in birds (Ohki et al., 1987), and the prominent role of the thymus in the induction of central tolerance was established by investigating the fate of developing thymocytes in response to superantigens (Kappler et al., 1987) or in T cell receptor (TCR) transgenic mice bearing a receptor for a HY-derived antigen (Kisielow et al., 1988). The identification of thymus-derived regulatory T cells (tTreg) (Sakagushi et al., 1995) completed our essential knowledge about the physiological function of the thymus, *i.e.*, the generation of a diverse repertoire of TCRs that are self-tolerant and competent against non-self. The central role of thymus-dependent central self-tolerance has now become a cornerstone of immune physiology (Mathis and Benoist, 2004; Kyewski and Klein, 2006).

THYMUS ORGANOGENESIS

Epithelial cells of the thymic cortex (cTECs, including thymic "nurse" cells) and medulla (mTECs) constitute around 85% of the thymus parenchyme. Other cells of the thymic stroma—dendritic cells (DCs) and macrophages—derive from the bone marrow. TECs arise from the endoderm of the 3rd pharyngeal pouch that, at around day 9 of embryonic development in the mouse (E9.0), contains thymic common epithelial progenitors (Blackburn and Manley, 2004; Anderson et al., 2007). Further development of this primitive epithelial rudiment between E9.5 and E12.5 depends on mesenchyme derived from the cephalic neural crest, the role of which extends beyond the early stages of thymus organogenesis (Foster et al., 2008).

Some human diseases and animal models involve a defect in thymus development, which leads to primary immune deficiencies. Di George's syndrome associates congenital absence or hypoplasia of the thymus and parathyroid glands together with defects in the heart and truncal vessels. This syndrome results partly from a migration failure of the cephalic neural crest. More recently, transgenic mouse models have revealed some of the molecular mechanisms involved in thymus development. Thus, *Hoxa3*$^{-/-}$ mice present thymic aplasia, parathyroid hypoplasia, that are often accompanied by defects of the heart and the great truncal vessels (Manley and Capecchi, 1995). In mice, the "nude" phenotype results from mutations in the *nude* gene locus on chromosome 11 that encodes the transcription factor winged-helix nude (*whn*) or forkhead box N1 (*Foxn1*) (Nehls et al., 1994). *Foxn1* is a master regulator gene of the TEC differentiation programme; it controls lineage progression in cTECs and mTECs, and regulates a series of genes implicated in TEC function, although is not essential for medullary sublineage divergence (Nowell et al., 2011). Importantly, the genetic mechanisms responsible for murine thymus organogenesis are conserved in humans (Farley et al., 2013) and this is important with regard to current attempts to regenerate the thymus in aging, as well as in many other clinical conditions associated with compromised immune function (Holländer et al., 2010). Indeed, contrary to earlier dogma, the thymus is functional until an advanced age, and thymopoiesis plays a central role in immune reconstitution after intensive chemotherapy or anti-retroviral treatment (Douek et al., 1998).

OVERVIEW OF T CELL DIFFERENTIATION IN THE THYMUS

The T cell immune system may be regarded as a 6th sensory organ that responds to different kinds of danger signals that are not detected by nerve cells. From the fetal liver, and later from the bone marrow, T lymphocyte progenitors migrate through the boundary between cortex and medulla into the thymic epithelial rudiment in response to chemoattractant factors (Wilkinson et al., 1999). They undergo many mitotic divisions in the outer cortex, and then differentiate after presentation of peptides by major histocompatibility complex (MHC) proteins expressed by thymic antigen-presenting cells (essentially cTECs, mTECs, DCs, and macrophages). The stochastic rearrangement of related β– then α-chains generates an enormous diversity of TCRs, many of which can bind self-antigen/MHC complexes with high affinity and are subsequently deleted (negative selection). Clonal deletion of thymocytes can occur both in the cortex and in the medulla (McCaughtry et al., 2008), but is most effective in the medulla.

Self-antigen presentation by thymic MHC proteins determines the whole process of T cell differentiation, which includes three alternative and exclusive fates for developing thymocytes: (1) Negative selection of self-reactive T cells generated during the random generation of TCR diversity due to the activity of the two recombination-activating enzymes RAG-1 and RAG-2; (2) Generation of self-specific tTreg cells; and (3) Positive selection of CD4+ and CD8+ effector and self-tolerant T cells. The first two events establish the establishment of the central arm of self-tolerance, while the avidity-affinity of the TCR—self-antigen—MHC interaction determines T cell negative or positive selection (Ashton-Rickardt et al., 1994). A remaining question is how the same MHC—self-peptide complexes can mediate either negative selection of self-reactive T cells or generation of self-specific tTreg cells (Klein et al., 2009; Stritesky et al., 2012). Another crucial question concerns the precise biochemical nature of self-peptides that are presented in the thymus, in particular during fetal life.

NEUROHYPOPHYSIAL FAMILY-RELATED PEPTIDES AND RECEPTORS IN THE THYMUS NETWORK

At the beginning of the 20th century, English scientists reported the galactogogue activity of thymus and corpus luteum extracts after their injection into the goat (Ott and Scott, 1910). At that time, oxytocin (OT) had not yet been identified as the specific factor of galactokinesis, which would be established in the late 50's by Vincent du Vigneaud and his team. In 1986, human thymus extracts were shown to possess potent uterine oxytocic activity, and OT was identified as the dominant member of the neurohypophysial family synthesized by TECs and thymic nurse cells in humans and different animal species (Geenen et al., 1986). Thymic T cells also express the OT receptor (OTR) and the V1b (or V3) vasopressin (AVP) receptor, which transduce nanomolar concentrations of OT and, accordingly to the rules of signaling

induced by OTR and V1b, these receptors transduce low concentrations of OT and AVP via the phosphoinositide pathway in mitogenic signals for freshly isolated thymocytes (reviewed in Martens et al., 1996a,b). Together, these data showed the existence of a functional signaling within the thymus mediated by OT synthesized by TECs acting on functional OTR and V1b receptors expressed by developing T cells. Further molecular characterization of the neurohypophysial receptors present in the murine thymus showed that *Otr* is transcribed by all thymic T cell subsets, while *V1b* is expressed only in double positive CD4+8+ and single positive CD8+ T cells (Hansenne et al., 2005). Because intrathymic OT concentrations are in agreement with the high affinity Kd (10^{-9}–10^{-10} M) of neurohypophysial receptors expressed by pre-T cells, the physico-chemical conditions are conducive to a functional signaling within the thymus network, which is not the case for the blood-borne OT and AVP, due to their low plasma concentration (10^{-11}–10^{-12} M).

Despite the strong evidence for the presence of neurohypophysial ligands and receptors in the thymus network, no secretion of OT or neurophysin could be detected in primary cultures of freshly isolated human TECs. Moreover, in the murine thymus, OT is not located in secretory granules but is diffuse in cytosol, in vesicles of the endoplasmic reticulum, and associated with keratin filaments (Wiemann and Ehret, 1993). This posed a fundamental problem, since the neuropeptide OT had been the basis for the development of the neurosecretion model by the Sharrer's in the 40′s. However, in 1990, Funder proposed a model of cell-to-cell cryptocrine (hidden secretion) signaling to characterize the direct membrane-to-membrane exchange of information between large epithelial nursing cells (like TECs in the thymus and Sertoli cells in the testis) and immature elements that migrate and differentiate at their contact (respectively, thymocytes and spermatocytes).

We thus proposed that, in the thymus, OT mediates a cryptocrine signaling between TECs and pre-T cells (Geenen et al., 1991). The observation of numerous points of focal adhesion between OT+ TECs and immature T cells (Wiemann and Ehret, 1993) prompted us to investigate the hypothesis that thymic OT could stimulate the activation of focal adhesion kinases (FAKs) in thymocytes. Among the proteins phosphorylated by OT in murine pre-T cells, two were precipitated with an anti-FAK mAb: one was identified as the $p125^{FAK}$, while the other was a coprecipitating 130-kD protein (most probably $p130^{Cas}$). In pre-T cells, OT also phosphorylates paxillin, a 68-kD protein located at focal adhesion sites and associated with $p125^{FAK}$ (Martens et al., 1998). Together, these data establish the existence of a functional OT-mediated cryptocrine signaling in the thymus network. The OT-mediated promotion of focal adhesion may contribute to the establishment of immunological "synapses" between TECs and immature T cells, which is fundamental for the completion of the T cell differentiation programme.

A PARADIGM SHIFT: FROM THYMIC NEUROPEPTIDES TO "NEUROENDOCRINE SELF-PEPTIDES"

As discussed above, self-antigen presentation by thymic MHC proteins is the essential mechanism that determines the whole process of T cell differentiation. Because OT and its associated neurophysin are self-peptides synthesized in TECs, we hypothesized a processing of the thymic OT precursor that would be related to antigen presentation rather than classical neurosecretion. If true, such a processing would fit with the model of cryptocrine signaling, which implicates a membrane targeting of the ligand. Following affinity-chromatography with a mAb against the monomorphic part of human MHC class I molecules, we identified, in human TEC plasma membrane, a 55-kD protein that was labeled by both anti-MHC class I mAb and a specific anti-neurophysin antiserum. Because this antiserum does not cross-react either with MHC class I proteins, or with β2-microglobulin, this 55-kD membrane protein could represent a hybrid protein including a neurophysin domain (10 kD) and a MHC class I heavy chain-related domain (45 kD). The formation of this hybrid could reside at the post-transcriptional level (such as a trans-splicing event) or at the post-translational level (such as ATP-dependent ubiquitinylation). The MHC class I domain could be implicated in the membrane targeting of the hybrid, whereas the neurophysin domain would bind OT for final presentation to pre-T cells. According to this explanation, the neurophysin part of the OT precursor could fulfill the same function in the thymus and in the hypothalamo-neurohypophysial axis: binding of the peptide OT and transport to the external limit of TECs or magnocellular neurons, respectively. The tyrosine residue in position 2 of OT plays an important role in its binding to neurophysin (Griffin et al., 1973) and, interestingly, the tyrosine residue in the same position plays a crucial role in the binding of antigens to some MHC class I alleles for their presentation (Maryanski et al., 1991).

The antigenic behavior of thymic OT is also supported by another type of experiment. The recognition of OT by specific mAbs at the outer surface of TEC plasma membrane induces a marked increase in the secretion of interleukin 6 (IL-6) and leukemia inhibitory factor (LIF) in the supernatant of human TEC primary cultures (Martens et al., 1996a,b). Given the nature of the specific epitopes recognized by anti-OT mAbs, it was concluded that OT is fully processed at the level of the TEC plasma membrane. The absence of any effect of anti-AVP mAbs further supports the conclusion that thymic OT behaves as the self-peptide of the neurohypophysial family.

Because OT is a cyclic nonapeptide, and since antigen presentation mostly concerns linear sequences, the hypothesis of a similar processing was investigated with the linear neurotensin (NT). Primary cultures of human TECs contain around 5 ng of $NT/10^6$ cells, of which 5% is associated with TEC plasma membranes. Again, no NT was detected in the supernatant of human TEC cultures. High performance liquid chromatography analysis of NT present in human TEC revealed a major peak of NT corresponding to intact NT_{1-13}. Using the same affinity column prepared with an anti-MHC class I mAb, NT, and NT-related peptides were retained on the column and were eluted together with MHC class I proteins (Vanneste et al., 1997). The C-terminal sequence of NT includes tyrosine, isoleucine, and leucine residues that all can be used to anchor most of MHC class I alleles. Thus, NT and NT-derived C-terminal fragments could be natural ligands for MHC class I alleles.

At this point and in association with the characterization of the thymic peptides related to different neuroendocrine families (Geenen et al., 1998), the biochemical nature and properties of the *"neuroendocrine self"* could be defined according to the following features:

1. Neuroendocrine self-peptides are the dominant members of neuroendocrine gene/protein families that are expressed in TECs of many species and usually correspond to sequences that have been highly conserved during the evolution of a given family.
2. A hierarchy characterizes their expression profile. In the neurohypophysial family, OT is the dominant peptide expressed in TECs from different species. In the tachykinin family, neurokinin A (NKA)—but not substance P—is the peptide generated from the processing in TECs of the preprotachykinin A gene product (Ericsson et al., 1990). In the insulin family, all members are expressed in the thymus network: IGF-2 (cTECs and mTECs) > IGF-1 (cTECs, mTECs, and macrophages) >> Insulin (rare subsets of mTECs). This hierarchical pattern is important, because the strength of immunological tolerance to a protein/peptide is proportional to its concentration in the thymus (Ashton-Rickardt et al., 1994). Blocking thymic IGF-mediated signaling at the level of IGF ligands (in particular IGF-2) or IGF receptors interferes with the early stages of T cell differentiation, while one mAb to proinsulin did not exert any significant effect (Kecha et al., 2000).
3. The autoimmune regulator gene/protein (AIRE) controls the degree of intrathymic transcription of the genes encoding neuroendocrine self-peptides (Anderson et al., 2002).
4. In the thymus, neuroendocrine precursors are not processed according to the model of neurosecretion but undergo antigenic processing for presentation by—or in association with—MHC proteins. This processing differs between thymic antigen-presenting cells (APCs) and professional APCs (DCs, B cells, and macrophages) in the periphery. For some neuroendocrine self-peptides (OT and NT), such differences imply that their presentation in the thymus is not tightly restricted by MHC alleles as much as presentation of nonself antigens and autoantigens by dedicated APCs in the periphery.
5. For some precursors of neuroendocrine self-peptide precursors, their transcription in TECs precedes their orthotopic expression in peripheral neuroendocrine glands/cells (Hansenne et al., 2005).

Therefore, depending on their behavior either as the source of neuroendocrine self-peptides or cryptocrine signals, the thymic repertoire of neuroendocrine precursors recapitulates at the molecular level the dual role of the thymus in T cell negative and positive selection.

INTEGRATED COEVOLUTION OF THE NEUROENDOCRINE AND IMMUNE SYSTEMS (FIGURE 1)

Throughout evolution, the neuroendocrine and innate immune systems have evolved in parallel, and coexist in all animal species without any apparent aggression of the innate immune system toward neuroendocrine glands. Indeed, Toll-like receptors, which are the essential mediators of innate response, do not react against normal or undamaged self. Some anticipatory immune responses are present even in jawless vertebrates (agnathans), mediated by

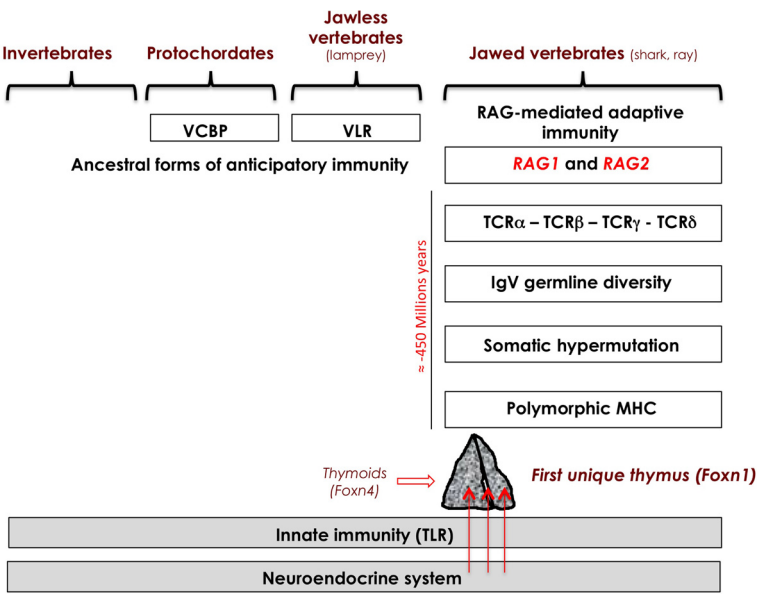


FIGURE 1 | Integrated evolution of the immune and neuroendocrine systems. Neuroendocrine principles are evolutionarily ancient and did not evolve extensively except by gene duplication and differential RNA splicing. A high risk of inherent autoimmunity toward the neuroendocrine tissues resulted from the appearance of RAG-dependent adaptive immunity in jawed cartilaginous fishes. Preceded by ancestor thymoids in lamprey larvae, the first unique thymus emerged in jawed vertebrates, and the intrathymic presentation of neuroendocrine self-peptides (arrows) may be viewed *a posteriori* as a very efficient and economical way to instruct the adaptive T cell system in tolerating neuroendocrine functions as early as during thymus-dependent cell differentiation. VCBP, variable region-containing chitin-binding protein; VLR, variable lymphocyte receptor.

diverse variable lymphocyte receptors (VLRs), with 4–12 leucine-rich repeat modules assembled by some gene conversion process. Some 500 million years ago, the emergence of transposon-like genes coding for recombination-activating enzymes RAG-1 and RAG-2 in cartilaginous fishes (sharks and rays, mainly) initiated the development of adaptive immunity (Agrawal et al., 1998; Boehm and Bleul, 2007; Hirano et al., 2011). The appearance of these two genes in the genome of gnathostomes (probably via horizontal transmission), and the subsequent appearance of the combinatorial immune system has been sometimes described as the "Big Bang" of immunology. Gene recombination in somatic lymphoid cells is responsible for the stochastic generation of diverse immune receptors for antigen, B-cell receptors (BCRs, $\pm 5 \times 10^{13}$ combinations) and T-cell receptors (TCRs, $\pm 10^{18}$ combinations). This extreme diversity of antigen receptors was directly associated with a high inherent risk of autoxicity that threatened survival of both species and individuals. This evolutionary pressure was so strong that, in accordance with Paul Ehrlich's concept of *horror autotoxicus*, novel structures, and mechanisms appeared with the specific role of protecting self against potential autoimmune attacks and orchestrating immunological self-tolerance. The first unique thymus also appeared in jawed cartilaginous fishes, concomitantly or very shortly after the emergence of adaptive immunity. The thymus did not abruptly appear but, as elegantly demonstrated by Bajoghli et al. (2011), was preceded by thymus-like lymphoepithelial structures in the gill baskets of lamprey larvae. These "thymoids" express the gene encoding forkhead box N4 (*Foxn4*), the ortholog of *Foxn1*, the transcription factor responsible for the differentiation of TECs in higher vertebrates as discussed above. *Foxn1* thus stands at a crucial place in the emergence of thymus epithelium that is essential for the control of T cell differentiation and self-tolerance programming (Boehm, 2011). The same study also provided strong evidence for a functional analogy between VLR assembly in thymoids and TCR recombination in the thymus, opening the hypothesis of autoimmune-like phenomena in jawless vertebrates.

The hierarchic organization of the thymic repertoire of neuroendocrine self-peptides is also very significant from an evolutionary point of view. Because major neuroendocrine principles had been established before the emergence of the anticipatory adaptive immune response, they had to be protected from the risk of autoimmunity inherent to this immune lottery. OT is a hypothalamic nonapeptide that is closely implicated at different steps of the reproductive process, from social affiliation and bonding to control of parturition and lactation. Thus, OT is fundamental for the preservation of animal and human species. Through its dominant expression in TECs, OT is much more tolerated than AVP, its hypothalamo-neurohypophysial homolog, which mainly controls water homeostasis and vascular tone. Interestingly, rare cases of autoimmune hypothalamitis with AVP deficiency and diabetes insipidus have been repeatedly observed (de Bellis et al., 2004), but no autoimmunity against OT has ever been reported. Similarly in the insulin family, insulin is the primary autoantigen of type 1 diabetes (T1D) while there is no report of autoimmunity against IGF-2, a growth factor fundamental for fetus development and individual ontogeny. However, because of their close homology, however, thymic neuroendocrine self-peptides promote immunological cross-tolerance to their whole family, and tolerance to insulin is indeed lower in $Igf2^{-/-}$ than in wild-type mice (Hansenne et al., 2006). Despite IGF-2 ubiquitous expression in extrathymic tissues, the deletion of *Igf2* in murine *Foxn1*+ TECs is also associated with a significant decrease in immunological tolerance to IGF-2 and to insulin (unpublished observations).

A DEFECT OF CENTRAL SELF-TOLERANCE AS THE PRIMARY EVENT DRIVING DEVELOPMENT OF AUTOIMMUNITY (FIGURE 2)

The evidence that T cells are programmed to recognize and tolerate the whole insulin family during their differentiation in the thymus prompted us to investigate the hypothesis of a defect in this education process as a potential source of self-reactive T cells directed specifically against insulin-secreting β cells of the pancreas. In other words, instead of considering autoimmune T1D as pathology of the pancreas, should we now consider the thymus as the primary defective organ? Already in 1973, Burnet theorized that the pathogenesis of autoimmune diseases first depends on a failure of self-tolerance and the appearance of "forbidden" self-reactive immune clones in the peripheral repertoire (Burnet, 1973), and even before the development of transgenic mice, a number of studies had provided elegant data supporting this hypothesis. Neonatal thymectomy prevents the emergence of autoimmune diabetes in an animal model of human T1D, the bio-breeding (BB) rat (Like et al., 1982). This benefit of neonatal thymectomy may now be explained by the removal of a defective thymus responsible for the continuous release and peripheral enrichment of the peripheral T cell repertoire with "forbidden" intolerant self-reactive T cells. Conversely, transplantation of thymus from diabetes-resistant (BBDR) to diabetes-prone BB (BBDR) rats prevents autoimmune diabetes in the latter (Georgiou and Bellgrau, 1989). Grafts of pure TECs from NOD mouse embryos to newborn C57BL/6 athymic nude mice also induce CD4 and CD8 T cell-mediated insulitis and sialitis (Thomas-Vaslin et al., 1997). Central tolerance and intrathymic apoptosis of self-reactive T cells were suspected to be defective in the NOD thymus (Kishimoto and Sprent, 2001; Zucchelli et al., 2005). However, a very careful recent study has established that thymic negative selection is functional in NOD mice (Mingueneau et al., 2012).

Transcription of all insulin-related genes has been investigated in the thymus of BBDR and BBDP rats. *Ins* and *Igf1* transcripts were detected in all BBDP and BBDR thymi whereas *Igf2* transcripts were also detected in all BBDR thymi but in only in 4 of 15 BBDP thymi. Such a defect of *Igf2* transcription in BBDP thymus could contribute both to their lymphopenia (including CD8+ T and RT6+ Treg cells) and to the absence of central tolerance to islet β cells (Kecha-Kamoun et al., 2001). In humans, *INS* transcripts are measured at a lower level in the thymus from fetuses with short class I variable number of tandem repeat (VNTR) alleles, the second genetic trait (*IDDM2*) of T1D susceptibility (Pugliese et al., 1997; Vafiadis et al., 1997). A number of other genetic loci associated with susceptibility to T1D could certainly determine disturbances in the

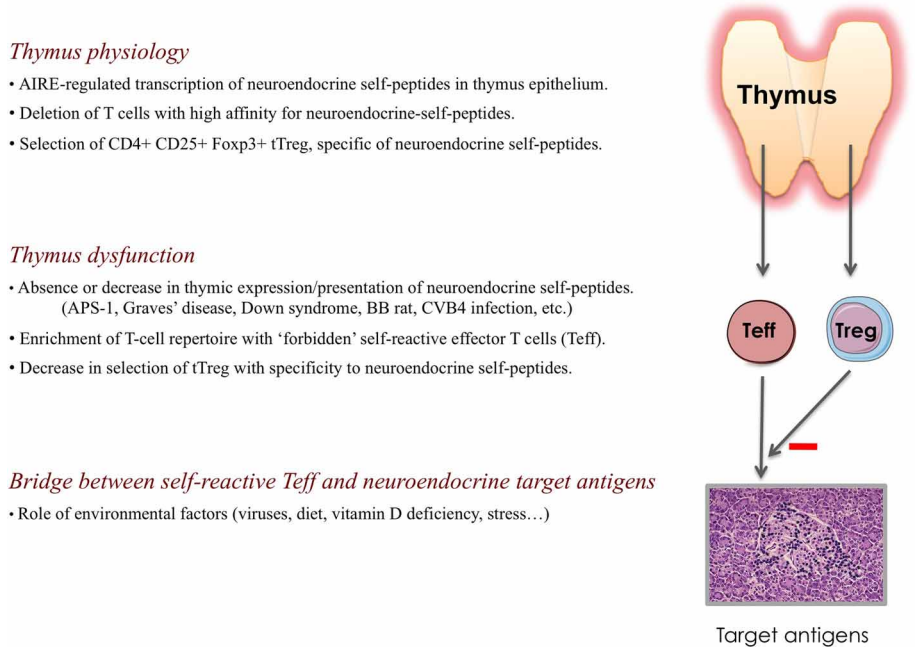


FIGURE 2 | Physiology of the thymus and the primary role of a thymus dysfunction in the development of autoimmune endocrine diseases. Throughout life, the thymus generates naïve T cells that are self-tolerant and competent against nonself-antigens, as well as self-specific thymus-dependent regulatory T (tTreg) cells. Under AIRE control, thymus epithelium (and mTECs particularly) transcribes genes encoding neuroendocrine self-peptides, as well as other tissue-restricted antigens. The absence or decrease in the intrathymic presentation of neuroendocrine self-peptides conducts to a continuous enrichment of the peripheral T cell repertoire with "forbidden" self-reactive T cells (Teff) bearing a TCR directed against neuroendocrine self-antigens, while thymic generation of specific tTreg cells is severely impaired. This thymus defect results in the absence of central self-tolerance to neuroendocrine tissues/cells, which is a condition necessary but not sufficient to initiate an autoimmune endocrine disease. Environmental factors also intervene in the promotion of a molecular bridge between self-reactive Teff and target neuroendocrine antigens.

thymus-dependent programming of central self-tolerance toward islet β cells.

In mice, where two genes code for (pro)insulin (*Ins1* and *Ins2*), *Ins2* is predominantly transcribed in rare subsets of mTECs while *Ins1* is dominantly expressed in murine islet β cells, which leads to a higher immunological tolerance to *Ins2*. This difference in the topography of *Ins2* and *Ins1* expression explains why breeding of $Ins2^{-/-}$ mice onto the NOD background markedly accelerates insulitis and diabetes onset (Thebault-Baumont et al., 2003), while the incidence of insulitis and autoimmune diabetes is considerably reduced in $Ins1^{-/-}$ congenic NOD mice (Moriyama et al., 2003). Susceptibility to autoimmune diabetes is also correlated with the level of *Ins2* transcription in the murine thymus (Chentoufi and Polychronakos, 2002). The role of thymic insulin in mediating central tolerance to islet β cells was demonstrated by the very rapid onset of autoimmune diabetes after a thymus-specific *Ins1* and *Ins2* deletion resulting from the crossing of $Ins1^{-/-}$ mice with others presenting a specific *Ins2* deletion in *Aire*-expressing mTECs (Fan et al., 2009). Of note, *Ins2* transcription in mTEC clones is not regulated by glucose (Levi and Polychronakos, 2009) and is increased about 20-fold by anti-lymphotoxin β mAb (Palumbo et al., 2006). *Ins* transcription in mTECs uses a start site different than in pancreatic islet β cells (Villasenor et al., 2008). In addition to Aire, the insulin transactivator MafA also induces *Ins* transcription in the thymus; targeted *Mafa* disruption reduces *Ins2* expression in the thymus and induces anti-islet autoantibodies (Noso et al., 2010).

The identification of the autoimmune regulator (*AIRE*) gene, a member of the zinc-finger gene family, led to an extremely important advancement in our knowledge of the central role played by a thymus dysfunction in the pathogenesis of organ-specific autoimmune diseases (The Finnish-German APECED Consortium, 1997). *AIRE* mutations are responsible for a rare recessive congenital syndrome called autoimmune polyglandular syndrome type 1 (APS-1) or Autoimmune Poly-Endocrinopathy, Candidiasis, Ectodermal Dystrophy (APECED) syndrome. *AIRE* encodes a protein with structural characteristics of a transcription factor: its transcription is maximal in thymus epithelium, and $Aire^{-/-}$ mice develop several autoimmune processes associated with a marked decrease in the intrathymic expression of numerous neuroendocrine self-peptides (Anderson et al., 2002). It also appears that both VNTR alleles (*IDDM2*) and the level of *AIRE* transcription in the thymus determine the concentration of *INS* transcripts in the human thymus (Sabater et al., 2005). The two plant homeodomains (PHDs) are critical regions for Aire function (Peterson et al., 2008); PHD2 strongly influences the ability of Aire to control the mTEC transcriptome and is therefore crucial for effective central tolerance induction (Yang et al., 2013). RANK signals from CD4+CD3− lymphoid tissue inducer (LTi) cells regulate

the development and differentiation of *Aire*-expressing mTECs (Rossi et al., 2007). Very recently, a mutual interdependence between Aire and microRNAs (miRs) was evidenced in the thymus since the profile of miR expression is severely affected in isolated murine and human mTECs from *Aire*$^{-/-}$ mice and since, in turn, *Aire* expression is downregulated in mTECs of Dicer null mutant mice (Macedo et al., 2012; Ucar et al., 2013).

An additional level of protection against autoimmunity is also provided by peripheral mechanisms of immunological tolerance that inactivate self-reactive T lymphocytes that have escaped the thymic censorship. Interestingly, extrathymic *Aire*-expressing cells were very recently identified as distinct bone marrow-derived tolerogenic cells that anergize effector self-reactive CD4+ T cells in secondary lymphoid organs (Gardner et al., 2008, 2013). These data further demonstrate that the Aire gene/protein is an essential shield against autoimmunity (Metzger and Anderson, 2011).

For several years, we have been investigating a novel hypothesis according which an infection by the enterovirus Coxsackie B4 (CVB4) could induce a thymus dysfunction and an impairment of central tolerance to the insulin protein/gene family. CVB4 can directly infect the epithelial and lymphoid compartments of the human and murine thymus, and promote a severe thymus dysfunction with massive pre-T cell depletion and a marked-up regulation of MHC class I expression by TECs and by double positive CD4+CD8+ immature thymic T cells (Brilot et al., 2002, 2004). CVB4 infection of murine fetal thymic organ cultures also interferes with T cell differentiation (Brilot et al., 2008). Outbred mice can be infected with CVB4 following oral inoculation, which results in systemic spreading of viral RNA and a prolonged detection of CVB4 RNA in thymus, spleen, and blood up to 70 days after inoculation (Jaïdane et al., 2006). Moreover, CVB4 infection of a murine mTEC line induces a dramatic decrease in *Igf2* mRNA and IGF-2 protein in this cell line, while *Igf1* transcripts were relatively unaffected. In this mTEC line, *Ins2* transcripts could not be detected (Jaïdane et al., 2012). Together, our data strongly suggest that CVB4 infection of the thymus could disrupt central self-tolerance to the insulin family, and could also enhance CVB4 virulence through induction of central tolerance to this virus.

With regard to autoimmune thyroiditis, which is the most frequent autoimmune endocrine disease, all major thyroid-specific antigens, i.e., thyroperoxydase, thyroglobulin, and thyrotropin receptor (TSHR), are also transcribed in human TECs in normal conditions (Paschke and Geenen, 1995; Sospedra et al., 1998). As first reported by Hammar, thymic hyperplasia is commonly observed in Graves' disease (Murakami et al., 1996), and homozygotes for an SNP allele predisposing to Graves' disease have significantly lower intrathymic *TSHR* transcripts than carriers of the protective allele (Colobran et al., 2011).

With regard to autoimmunity directed against peripheral nonendocrine organs, a defect in α-myosin expression in TECs was recently shown to exert a central role in the pathogenesis of autoimmune myocarditis in mice and humans (Lv et al., 2011; Lv and Lipes, 2012). Also, a defect in Aire-mediated central tolerance to myelin protein zero promotes the development of an autoimmune Th1 effector response toward peripheral nerves (Su et al., 2012).

THE CONCEPT OF "NEGATIVE/TOLEROGENIC SELF-VACCINATION"

Although *Ins2* is expressed at low levels in rare mTECs, thymic (pro)insulin has an essential role in protecting islet β cells against diabetogenic autoimmunity. However, insulin *per se* does not exert any significant tolerogenic properties that could be exploited to reprogram immunological tolerance to islet β cells. With the exception of two studies (Fourlanos et al., 2011; Roep et al., 2013), all the clinical trials based on insulin administration by different ways failed to preserve the residual β cell mass once the autoimmune attack has induced patent T1D. On the contrary, the potent immunogenic properties of insulin were revealed in different studies (Blanas et al., 1996; Liu et al., 2002), and this immunogenicity could be related to the very low level of insulin gene transcription in the thymus. Other studies have also evidenced the risk of hypersensitivity or anaphylaxis following administration of an autoantigen (Pedotti et al., 2001).

Nevertheless, the development of peptide-based therapeutic vaccines remains an attractive approach because of the specificity of immune suppression or regulation directed to specific pathogenic self-reactive T cells. In this context, infusion of a strongly active insulin mimetope was recently shown to convert naïve T cells into Foxp3+ Treg cells *in vivo* and to prevent autoimmune diabetes in NOD mice (Daniel et al., 2011). We proposed that IGF-2 could be a safer and more valuable basis for developing a specific "negative/tolerogenic self-vaccination," on the basis that *Igf2* transcription is defective in the thymus of BBDP rats (Kecha-Kamoun et al., 2001) and that IGF-2 mediates significant cross-tolerance to insulin (Hansenne et al., 2006). The concept of negative self-vaccination implies both the competition between IGF-2 and insulin-derived epitopes for presentation by DQ2 and DQ8 alleles, as well as a tolerogenic response—including recruitment of Treg cells–induced by MHC-presentation of IGF-2 self-peptides (Geenen et al., 2004, 2010).

CONCLUSION

Our studies have established that the thymus is not a classical neuroendocrine gland, but an obligatory intersection between the adaptive immune and neuroendocrine systems (Geenen, 2010). Moreover, they have elucidated how thymic epithelium is responsible for the programming of immunological central self-tolerance toward neuroendocrine functions through presentation of neuroendocrine self-peptides. They have also helped to resolve three major questions concerning the pathogenesis of autoimmune endocrine disorders: Why are the neuroendocrine glands so frequently tackled by an autoimmune process? What is the origin of the pathogenic effector self-reactive T cells? And what explains the tissue/cell-specificity of the autoimmune processes? There is no doubt that this novel knowledge will soon be exploited for the development of innovative strategies to prevent and cure a variety of organ-specific devastating autoimmune diseases such as T1D.

ACKNOWLEDGMENTS

Vincent Geenen is Research Director and Gwennaëlle Bodart is Research Assistant of the Belgian Fund for Scientific Research of Belgium (F.S.R.-NFSR). We are extremely grateful to Pr Gareth

Leng (University of Edinburgh) for his critical reading of this manuscript. These studies are or have been supported by F.S.R.-NFSR and the Fund for Research in Industry and Agronomy (FRIA, Belgium), by the Fund Leon Fredericq for biomedical research at the University Hospital of Liege, by the Special Research Fund of the University of Liege, by the Fund A. Rahier for research in diabetes, by Wallonia (Toledia, ThymUp and Raparray conventions), by the European Federation for the Study of Diabetes (EFSD, Düsseldorf), by the Juvenile Diabetes Research Foundation (JDRF, New York), and by the European Commission (Eurothymaide PF6 Integrated Project, contract LHSB-CT-2003-503410, www.eurothymaide.org).

REFERENCES

Agrawal, A., Eastman, Q. M., and Schatz, D. G. (1998). Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. *Nature* 394, 744–751. doi: 10.1038/29457

Anderson, G., Lane, P. J. L., and Jenkinson, E. J. (2007). Generating intrathymic microenvironments to establish T-cell tolerance. *Nat. Rev. Immunol.* 7, 954–962. doi: 10.1038/nri2187

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzin, S. P., Turley, S. J., et al. (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298, 1395–1401. doi: 10.1126/science.1075958

Ashton-Rickardt, P., Bandeira, A., Delaney, J. R., Van Kaer, L., Pircher, H. P., Zinkernagel, R. M., et al. (1994). Evidence for a differential avidity model of T cell selection in the thymus. *Cell* 76, 651–663. doi: 10.1016/0092-8674(94)90505-3

Bajoghli, B., Guo, P., Aghaallaei, N., Hirano, M., Strohmeier, C., McCurley, N., et al. (2011). A thymus candidate in lampreys. *Nature* 470, 90–94. doi: 10.1038/nature09655

Blackburn, C. C., and Manley, N. R. (2004). Developing a new paradigm for thymus organogenesis. *Nat. Rev. Immunol.* 4, 278–289. doi: 10.1038/nri1331

Blanas, E., Carbone, F. R., Allison, J., Miller, J. F. A. P., and Heath, W. R. (1996). Induction of autoimmune diabetes by oral administration of autoantigen. *Science* 274, 1707–1709. doi: 10.1126/science.274.5293.1707

Boehm, T. (2011). Design principles of adaptive immune systems. *Nat. Rev. Immunol.* 11, 307–317. doi: 10.1038/nri2944

Boehm, T., and Bleul, C. (2007). The evolutionary history of lymphoid organs. *Nat. Immunol.* 8, 131–135. doi: 10.1038/ni1435

Brilot, F., Chehadeh, W., Charlet-Renard, C., Martens, H., Geenen, V., and Hober, D. (2002). Persistent infection of human thymic epithelial cells by coxsackievirus B4. *J. Virol.* 76, 5260–5265. doi: 10.1128/JVI.76.10.5260-5265.2002

Brilot, F., Geenen, V., Hober, D., and Stoddart, C. (2004). Coxsackievirus B4 infection of human fetal thymus cells. *J. Virol.* 78, 9854–9861. doi: 10.1128/JVI.78.18.9854-9861.2004

Brilot, F., Jaïdane, H., Geenen, V., and Hober, D. (2008). Coxsackievirus B4 infection of murine foetal thymus organ cultures. *J. Med. Virol.* 80, 659–666. doi: 10.1002/jmv.21016

Burnet, F. M. (1973). A reassessment of the forbidden clone hypothesis of autoimmune diseases. *Aust. J. Exp. Biol. Med. Sci.* 50, 1–9. doi: 10.1038/icb.1972.1

Burnet, F. M., and Mackay, I. R. (1963). Lymphoepithelial structures and autoimmune disease. *Lancet* 2, 1030–1033.

Chentoufi, A. A., and Polychronakos, C. (2002). Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance. *Diabetes* 51, 1383–1390. doi: 10.2337/diabetes.51.5.1383

Colobran, R., del Pilar Armengol, M., Faner, R., Gärtner, M., Tykocinski, L. O., Lucas, A., et al. (2011). Association of an SNP with intrathymic transcription of TSHR and Graves' disease: a role for defective tolerance. *Hum. Mol. Genet.* 20, 3415–3423. doi: 10.1093/hmg/ddr247

Daniel, C., Weigmann, B., Bronson, R., and von Boehmer, H. (2011). Prevention of type 1 diabetes in mice by tolerogenic vaccination with a strong insulin mimetope. *J. Exp. Med.* 208, 1501–1510. doi: 10.1084/jem.20110574

de Bellis, A., Bizzarro, A., and Bellastella, A. (2004). "Autoimmune central diabetes insipidus," in *Immunoendocrinology in Health and Disease*, eds V. Geenen and G. P. Chrousos (New York, NY: Marcel Dekker), 461–490.

Douek, D. C., MacFarland, R. D., Keiser, P. H., Gage, E. A., Massey, J. M., Haynes, B. F., et al. (1998). Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396, 690–695. doi: 10.1038/25374

Ericsson, A., Geenen, V., Robert, F., Legros, J. J., Vrindts-Gevaert, Y., Franchimont, P., et al. (1990). Expression of preprotachykinin A and neuropeptide-Y messenger RNA in the thymus. *Mol. Endocrinol.* 4, 1211–1218. doi: 10.1210/mend-4-8-1211

Fan, Y., Rudert, W. A., Grupillo, M., He, J., Sisino, G., and Trucco, M. (2009). Thymus specific deletion of insulin induces autoimmune diabetes. *EMBO J.* 28, 2812–2824. doi: 10.1038/emboj.2009.212

Farley, A. M., Morris, L. X., Vroegindeweij, E., Depreter, M. L., Vaidya, H., Stenhouse, F. H., et al. (2013). Dynamics of thymus organogenesis and colonization in early human development. *Development* 140, 2015–2026. doi: 10.1242/dev.087320

Foster, K., Sheridan, J., Veiga-Fernandes, H., Roderick, K., Pachnis, V., Adams, R., et al. (2008). Contribution of neural crest-derived cells in the embryonic and adult thymus. *J. Immunol.* 180, 3183–3189.

Fourlanos, S., Perry, C., Gellert, S. A., Martinuzzi, E., Mallone, R., Butler, J., et al. (2011). Evidence that nasal insulin induces immune tolerance to insulin in adults with autoimmune diabetes. *Diabetes* 60, 1237–1245. doi: 10.2337/db10-1360

Funder, J. W. (1990). Paracrine, cryptocrine, acrocrine. *Mol. Cell. Endocrinol.* 70, C21–C24. doi: 10.1016/0303-7207(90)90052-A

Gardner, J. M., Devos, J. J., Friedman, R. S., Wong, D. J., Ta, Y. X., Zhou, X., et al. (2008). Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science* 321, 843–847. doi: 10.1126/science.1159407

Gardner, J. M., Metzger, T. C., McMahon, E. J., Au-Yeung, B. B., Krawisz, A. K., Lu, W., et al. (2013). Extrathymic Aire-expressing cells are a distinct bone marrow-derived population that induce functional activation of CD4+ T cells. *Immunity* 39, 560–572. doi: 10.1016/j.immuni.2013.08.005

Geenen, V. (2010). The thymus as an obligatory intersection between the immune and neuroendocrine systems: pharmacological implications. *Curr. Opin. Pharmacol.* 10, 406–407. doi: 10.1016/j.coph.2010.05.011

Geenen, V., Kecha, O., and Martens, H. (1998). Thymic expression of neuroendocrine self-peptide precursors: role in T cell survival and self-tolerance. *J. Neuroendocrinol.* 10, 811–822. doi: 10.1046/j.1365-2826.1998.00269.x

Geenen, V., Legros, J. J., Franchimont, P., Baudrihaye, M., Defresne, M. P., and Boniver, J. (1986). The neuroendocrine thymus: coexistence of oxytocin and neurophysin in the human thymus. *Science* 232, 508–511. doi: 10.1126/science.3961493

Geenen, V., Louis, C., Martens, H., and The Belgian Diabetes Registry. (2004). An insulin-like growth factor 2-derived self antigen inducing a regulatory cytokine profile after presentation to peripheral blood mononuclear cells form DQ8+ type 1 diabetic adolescents: preliminary design of a thymus-based tolerogenic self-vaccination. *Ann. N.Y. Acad. Sci.* 1037, 59–64. doi: 10.1196/annals.1337.008

Geenen, V., Mottet, M., Dardenne, O., Kermani, H., Martens, H., Francois, J. M., et al. (2010). Thymic self-antigens for the design of a negative/tolerogenic self-vaccination against type 1 diabetes. *Curr. Opin. Pharmacol.* 10, 461–472. doi: 10.1016/j.coph.2010.04.005

Geenen, V., Robert, F., Martens, H., Benhida, A., De Giovanni, G., Defresne, M. P., et al. (1991). At the cutting edge. Biosynthesis and paracrine/cryptocrine actions of 'self' neurohypophysial-related peptides in the thymus. *Mol. Cell. Endocrinol.* 76, C27–C31. doi: 10.1016/0303-7207(91)90277-Y

Georgiou, H. M., and Bellgrau, D. (1989). Thymus transplantation and disease prevention in the diabetes-prone Bio-Breeding rat. *J. Immunol.* 142, 3400–3405.

Griffin, G. H., Alazard, R., and Cohen, P. (1973). Complex formation between bovine neurophysin I and oxytocin, vasopressin and tripeptide analogs of their NH_2-terminal region. *J. Biol. Chem.* 248, 7975–7978.

Hammar, J. A. (1921). The new views as to the morphology of the thymus gland and their bearing on the problem of the function of the thymus. *Endocrinology* 5, 543–573. doi: 10.1210/endo-5-5-543

Hansenne, I., Charlet-Renard, C., Greimers, R., and Geenen, V. (2006). Dendritic cell differentiation and tolerance to insulin-related peptides in *Igf2*-deficient mice. *J. Immunol.* 176, 4651–4657.

Hansenne, I., Rasier, G., Péqueux, C., Brilot, F., Renard, Ch., Breton, C. et al. (2005). Ontogenesis and functional aspects of oxytocin and vasopressin gene expression in the thymus network. *J. Neuroimmunol.* 158, 67–75. doi: 10.1016/j.jneuroim.2004.08.007

Hirano, M., Das, S., Guo, P., and Cooper, M. D. (2011). The evolution of adaptive immunity in vertebrates. *Adv. Immunol.* 109, 125–157. doi: 10.1016/B978-0-12-387664-5.00004-2

Holländer, G. A., Krenger, W., and Blazar, B. R. (2010). Emerging strategies to boost thymic function. *Curr. Opin. Pharmacol.* 10, 443–453. doi: 10.1016/j.coph.2010.04.008

Jaïdane, H., Caloone, D., Lobert, P. E., Sane, F., Dardenne, O., Naquet, P., et al. (2012). Persistent infection of thymic epithelial cells with coxsackievirus B4 results in decreased expression of type 2 insulin-like growth factor. *J. Virol.* 86, 11151–11162. doi: 10.1128/JVI.00726-12

Jaïdane, H., Gharbi, J., Lobert, P. E., Lucas, B., Hiar, R., M'Hadheb, M. B., et al. (2006). Prolonged viral RNA detection in blood and lymphoid tissues from coxsackievirus B4-E2 orally-inoculated mice. *Microbiol. Immunol.* 50, 971–974.

Kappler, J. W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. *Cell* 49, 273–280. doi: 10.1016/0092-8674(87)90568-X

Kecha, O., Brilot, F., Martens, H., Franchimont, N., Renard, C., Greimers, R., et al. (2000). Involvement of insulin-like growth factors in early T cell development: a study using fetal thymic organ cultures. *Endocrinology* 141, 1209–1217. doi: 10.1210/en.141.3.1209

Kecha-Kamoun, O., Achour, I., Martens, H., Collette, J., Lefèbvre, P. J., Greiner, D. L., et al. (2001). Thymic expression of insulin-related genes in an animal model of type 1 diabetes. *Diabetes Metab. Res. Rev.* 17, 146–152. doi: 10.1002/dmrr.182

Kishimoto, H., and Sprent, J. (2001). A defect in central tolerance in NOD mice. *Nat. Immunol.* 2, 1025–1031. doi: 10.1038/ni726

Kisielow, P., Bluethmann, H., Staerz, U. D., Steinmetz, M., and von Boehmer, H. (1988). Tolerance in T cell receptor transgenic mice: deletion of nonmature CD4+8+ thymocytes. *Nature* 333, 742–746. doi: 10.1038/333742a0

Klein, L., Hinterberger, M., Wirnsberger, G., and Kyewski, B. (2009). Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat. Rev. Immunol.* 9, 833–844. doi: 10.1038/nri2669

Kyewski, B., and Klein, L. (2006). A central tole for central tolerance. *Annu. Rev. Immunol.* 24, 571–606. doi: 10.1146/annurev.immunol.23.021704.115601

Levi, D., and Polychronakos, C. (2009). Regulation of insulin gene expression by cytokines and cell-cell interactions in mouse medullary thymic epithelial cells. *Diabetologia* 52, 2151–2158. doi: 10.1007/s00125-009-1448-y

Like, A. A., Kislaukis, E., Williams, R. M., and Rossini, A. A. (1982). Neonatal thymectomy prevents spontaneous diabetes mellitus in the BB:W rat. *Science* 216, 644–646. doi: 10.1126/science.7041259

Liu, E., Moriyama, H., Abiru, N., Miao, D., Yu, L., Taylor, R. M., et al. (2002). Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides. *J. Clin. Invest.* 110, 1021–1027. doi: 10.1172/JCI215488

Lv, H., Havari, E., Pinto, S., Gottumukkala, R. V., Cornivelli, L., Raddassi, K., et al. (2011). Impaired thymic tolerance to a-myosin directs autoimmunity to the heart in mice and humans. *J. Clin. Invest.* 121, 1561–1573. doi: 10.1172/JCI44583

Lv, H. J., and Lipes, M. A. (2012). Role of impaired central tolerance to a-myosin in inflammatory heart disease. *Trends Cardiovasc. Med.* 22, 113–117. doi: 10.1016/j.tcm.2012.07.005

Macedo, C., Evangelista, A. F., Marques, M. M., Octavillio-Silva, S., Donadi, E. A., Sakamoto-Hojo, E. T., et al. (2012). Autoimmune regulator (Aire) controls the expression of microRNAs in medullary thymic epithelial cells. *Immunobiology* 218, 554–560. doi: 10.1016/j.imbio.2012.06.013

Manley, N. R., and Capecchi, M. R. (1995). The role of Hoxa-3 in mouse thymus and thyroid development. *Development* 121, 1989–2003.

Martens, H., Kecha, O., Charlet-Renard, C., Defresne, M. P., and Geenen, V. (1998). Neurohypophysial peptides stimulate the phosphorylation of pre-T cells focal adhesion kinases. *Neuroendocrinology* 67, 282–289. doi: 10.1159/000054324

Martens, H., Malgrange, B., Robert, F., Charlet, C., De Groote, D., Heymann, D., et al. (1996a). Cytokine production by human thymic epithelial cells: control by the immune recognition of the neurohypophysial self-antigen. *Regul. Pept.* 67, 39–45. doi: 10.1016/S0167-0115(96)00105-X

Martens, H., Goxe, B., and Geenen, V. (1996b). The thymic repertoire of neuroendocrine self-antigens: physiological implications in T-cell life and death. *Immunol. Today* 17, 312–317. doi: 10.1016/0167-5699(96)10023-2

Maryanski, J. L., Romero, P., Van Pel, A., Boon, T., Salemme, F. R., Cerrottini, J. C., et al. (1991). The identification of tyrosine as a common key residue in unrelated H-2K^d restricted antigenic peptides. *Int. Immunol.* 3, 1035–1042. doi: 10.1093/intimm/3.10.1035

Mathis, D., and Benoist, C. (2004). Back to central tolerance. *Immunity* 20, 509–516. doi: 10.1016/S1074-7613(04)00111-6

McCaughtry, T. M., Baldwin, T. A., Wilken, M. S., and Hogquist, K. A. (2008). Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *J. Exp. Med.* 205, 2575–2584. doi: 10.1084/jem.20080866

Metzger, T. C., and Anderson, M. S. (2011). Control of central and peripheral tolerance by Aire. *Immunol. Rev.* 241, 89–103. doi: 10.1111/j.1600-065X.2011.01008.x

Miller, J. F. A. P. (1961). Immunological function of the thymus. *Lancet* 2, 748–749. doi: 10.1016/S0140-6736(61)90693-6

Mingueneau, M., Jiand, W., Feuerer, M., Mathis, D., and Benoist, C. (2012). Thymic negative selection is functional in NOD mice. *J. Exp. Med.* 209, 623–637. doi: 10.1084/jem.20112593

Moriyama, H., Abiru, N., Paronen, J., Sikora, K., Liu, E., Miao, D., et al. (2003). Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10376–10381. doi: 10.1073/pnas.1834450100

Murakami, M., Hosoi, Y., Negishi, T., Kamiya, Y., Miyashita, K., Yamada, M., et al. (1996). Thymic hyperplasia in patients with Graves' disease. Identification of thyrotropin receptors in human thymus. *J. Clin. Invest.* 98, 2228–2234. doi: 10.1172/JCI119032

Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H., and Boehm, T. (1994). New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372, 103–106. doi: 10.1038/372103a0

Noso, S., Kataoka, K., Kawabata, Y., Babaya, N., Hiromine, Y., Yamaji, K., et al. (2010). Insulin transactivator MafA regulates intrathymic expression of insulin and affects susceptibility to type 1 diabetes. *Diabetes* 59, 2579–2587. doi: 10.2337/db10-0476

Nowell, C. S., Bredenkamp, N., Tetélin, S., Jin, X., Tischner, C., Vaidya, H., et al. (2011). Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence. *PLoS Genet.* 7:e1002348. doi: 10.1371/journal.pgen.1002348

Ohki, H., Martin, C., Corbel, C., Coltey, M., and Le Douarin, N. M. (1987). Tolerance induced by thymic epithelial grafts in birds. *Science* 237, 1032–1035. doi: 10.1126/science.3616623

Ott, I., and Scott, J. C. (1910). The galactogogue action of the thymus and corpus luteum. *Proc. Soc. Exp. Biol. Med.* 8, 49–53. doi: 10.3181/00379727-8-28

Palumbo, M. O., Levi, D., Chentoufi, A. A., and Polychronakos, C. (2006). Isolation and characterisation of proinsulin-producing medullary thymic epithelial cells clones. *Diabetes* 55, 2595–2601. doi: 10.2337/db05-1651

Paschke, R., and Geenen, V. (1995). Messenger RNA expression for a TSH receptor variant in the thymus of a two-year old child. *J. Mol. Med.* 73, 577–580. doi: 10.1007/BF00195143

Pedotti, R., Mitchell, D., Wedemeyer, J., Karpuj, M., Chabas, D., Hattab, E. M., et al. (2001). An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat. Immunol.* 2, 216–222. doi: 10.1038/85266

Peterson, P., Org, T., and Rebane, A. (2008). Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. *Nat. Rev. Immunol.* 8, 948–957. doi: 10.1038/nri2450

Pugliese, A., Zeller, M., Fernandez, A. Jr., Zalcberg, L. J., Barnett, R. J., Ricordi, C., et al. (1997). The insulin gene is transcribed in the human thymus and transcription levels correlate with allelic variation at the *INS* VNTR-*IDDM2* susceptibility

locus for type 1 diabetes. *Nat. Genet.* 15, 293–297. doi: 10.1038/ng0397-293

Roep, B. O., Solvason, N., Gottlieb, P. A., Abreu, J. R., Harrison, L. C., Eisenbarth, G. S., et al. (2013). Plasmid-encoding proinsulin preserves C-peptide while specifically reducing proinsulin-specific CD8+ T cells in type 1 diabetes. *Sci. Transl. Med*. 5, 191ra82. doi: 10.1126/scitranslmed.3006103

Rossi, S. W., Kim, M. Y., Leibbrandt, A., Parnell, S. M., Jenkinson, W. E., Glanville, S. H., et al. (2007). RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J. Exp. Med*. 204, 1267–1272. doi: 10.1084/jem.20062497

Sabater, L., Ferrer-Francesch, X., Sospedra, M., Caro, P., Juan, M., and Pujol-Borrell, R. (2005). Insulin alleles and autoimmune regulator (AIRE) gene both influence insulin expression in the thymus. *J. Autoimmun*. 25, 312–318. doi: 10.1016/j.jaut.2005.08.006

Sakagushi, S., Sakagushi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164.

Selye, H. (1941). Variations in organ size caused by chronic treatment with adrenal cortical compounds. An example of a dissociated adaptation syndrome. *J. Anat*. 76, 94–99.

Sospedra, M., Ferrer-Francesch, X., Dominguez, O., Juan, M., Foz-Sala, M., and Pujol-Borrell, R. (1998). Transcription of a broad range of self-antigens in human thymus suggests a role for central mechanisms in tolerance toward peripheral antigens. *J. Immunol*. 161, 5918–5929.

Stritesky, G. L., Jameson, S. C., and Hogquist, K. A. (2012). Selection of self-reactive T cells in the thymus. *Annu. Rev. Immunol*. 30, 95–114. doi: 10.1146/annurev-immunol-020711-075035

Su, M. A., Davini, D., Cheng, P., Giang, K., Fan, U., DeVoss, J. J., et al. (2012). Defective autoimmune regulator-dependent central tolerance to myelin protein zero is linked to autoimmune peripheral neuropathy. *J. Immunol*. 188, 4906–4912. doi: 10.4049/jimmunol.1200493

Thebault-Baumont, K., Dubois-Laforgue, D., Krief, P., Briand, J. P., Halbout, P., Vallon-Geoffroy, K., et al. (2003). Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice. *J. Clin. Invest.* 111, 851–857. doi: 10.1172/JCI16584

The Finnish-German Consortium. (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat. Genet*. 17, 399–403. doi: 10.1038/ng1297-399

Thomas-Vaslin, V., Damotte, D., Coltey, M., Le Douarin, N. M., Coutinho, A., and Salaün, J. (1997). Abnormal T cell selection on NOD thymic epithelium is sufficient to induce autoimmune manifestations in C57/BL/6 athymic nude mice. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4598–4603. doi: 10.1073/pnas.94.9.4598

Ucar, O., Tykocinski, L.-O., Dooley, J., Liston, A., and Kyewski, B. (2013). An evolutionary conserved mutual interdependence between Aire and microRNAs in promiscuous gene expression. *Eur. J. Immunol.* 43, 1769–1778. doi: 10.1002/eji.201343343

Vafiadis, P., Bennett, S. T., Todd, J. A., Nadeau, J., Grabs, R., Goodyer, C. G., et al. (1997). Insulin expression in human thymus is modulated by *INS* VNTR alleles at the *IDDM2* locus. *Nat. Genet.* 15, 289–292. doi: 10.1038/ng0397-289

Vanneste, Y., Ntodou Thome, A., Vandersmissen, E., Charlet, C., Franchimont, D., Martens, H., et al. (1997). Identification of neurotensin-related peptides in human thymic epithelial cell membranes and relationship with major histocompatibility complex class I molecules. *J. Neuroimmunol*. 76, 161–166. doi: 10.1016/S0165-5728(97)00052-0

Villasenor, J., Besse, W., Benoist, C., and Mathis, D. (2008). Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15854–15859. doi: 10.1073/pnas.0808069105

Wiemann, M., and Ehret, G. (1993). Subcellular localization of immunoreactive oxytocin within thymic epithelial cells of the male mouse. *Cell Tissue Res*. 273, 79–87. doi: 10.1007/BF00304614

Wilkinson, B., Owen, J. J. T., and Jenkinson, E. J. (1999). Factors regulating stem cell recruitment to the fetal thymus. *J. Immunol*. 162, 3873–3881.

Yang, S., Bansal, K., Lopes, J., Benoist, C., and Mathis, D. (2013). Aire's plant homeodomain (PHD)-2 is critical for induction of immunological tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 110, 1833–1838. doi: 10.1073/pnas.1222023110

Zucchelli, S., Holler, P., Yamagata, T., Roy, M., Benoist, C., and Mathis, D. (2005). Defective central tolerance induction in NOD mice: genomics and genetics. *Immunity* 22, 385–396. doi: 10.1016/j.immuni.2005.01.015

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 July 2013; paper pending published: 21 August 2013; accepted: 27 September 2013; published online: 16 October 2013.

*Citation: Geenen V, Bodart G, Henry S, Michaux H, Dardenne O, Charlet-Renard C, Martens H and Hober D (2013) Programming of neuroendocrine self in the thymus and its defect in the development of neuroendocrine autoimmunity. Front. Neurosci. **7**:187. doi: 10.3389/fnins.2013.00187*

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in
NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 10 October 2013
doi: 10.3389/fnins.2013.00183

Brain immune interactions and air pollution: macrophage inhibitory factor (MIF), prion cellular protein (PrP^C), Interleukin-6 (IL-6), interleukin 1 receptor antagonist (IL-1Ra), and interleukin-2 (IL-2) in cerebrospinal fluid and MIF in serum differentiate urban children exposed to severe vs. low air pollution

Lilian Calderón-Garcidueñas[1,2]*, Janet V. Cross[3], Maricela Franco-Lira[2], Mariana Aragón-Flores[2], Michael Kavanaugh[1], Ricardo Torres-Jardón[4], Chih-kai Chao[1], Charles Thompson[1], Jing Chang[5], Hongtu Zhu[5] and Amedeo D'Angiulli[6]

[1] *Department of Biomedical Sciences, The Center for Structural and Functional Neurosciences, The University of Montana, Missoula, MT, USA*
[2] *Hospital Central Militar, Secretaria de la Defensa Nacional, Mexico City, Mexico*
[3] *Department of Pathology, School of Medicine, University of Virginia, Charlottesville, VA, USA*
[4] *Centro de Ciencias de la Atmósfera, Universidad Nacional Autónoma de México, Mexico City, Mexico*
[5] *Department of Biostatistics, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC, USA*
[6] *Department of Neuroscience, Carleton University, Ottawa, ON, Canada*

Edited by:
Adam Denes, University of Manchester, UK

Reviewed by:
Zsuzsanna Környei, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Hungary
Muhammad Sajjad, University of Bonn, Germany
Jesus M. Pradillo, University Complutense of Madrid, Technical University of Madrid, Spain

***Correspondence:**
Lilian Calderón-Garcidueñas, The Center for Structural and Functional Neurosciences, The University of Montana, 32 Campus Drive, Skaggs Building 287, Missoula, MT 59812, USA
e-mail: lilian.calderon-garciduenas@umontana.edu

Mexico City Metropolitan Area children chronically exposed to high concentrations of air pollutants exhibit an early brain imbalance in genes involved in oxidative stress, inflammation, innate and adaptive immune responses along with accumulation of misfolded proteins observed in the early stages of Alzheimer and Parkinson's diseases. A complex modulation of serum cytokines and chemokines influences children's brain structural and gray/white matter volumetric responses to air pollution. The search for biomarkers associating systemic and CNS inflammation to brain growth and cognitive deficits in the short term and neurodegeneration in the long-term is our principal aim. We explored and compared a profile of cytokines, chemokines (Multiplexing LASER Bead Technology) and Cellular prion protein (PrP^C) in normal cerebro-spinal-fluid (CSF) of urban children with high vs. low air pollution exposures. PrP^C and macrophage inhibitory factor (MIF) were also measured in serum. Samples from 139 children ages 11.91 ± 4.2 years were measured. Highly exposed children exhibited significant increases in CSF MIF ($p = 0.002$), IL6 ($p = 0.006$), IL1ra ($p = 0.014$), IL-2 ($p = 0.04$), and PrP^C ($p = 0.039$) vs. controls. MIF serum concentrations were higher in exposed children ($p = 0.009$). Our results suggest CSF as a MIF, IL6, IL1Ra, IL-2, and PrP^C compartment that can possibly differentiate air pollution exposures in children. MIF, a key neuro-immune mediator, is a potential biomarker bridge to identify children with CNS inflammation. Fine tuning of immune-to-brain communication is crucial to neural networks appropriate functioning, thus the short and long term effects of systemic inflammation and dysregulated neural immune responses are of deep concern for millions of exposed children. Defining the linkage and the health consequences of the brain / immune system interactions in the developing brain chronically exposed to air pollutants ought to be of pressing importance for public health.

Keywords: Alzheimer, air pollution, children, innate immunity, neurodegeneration, neuroinflammation, particulate matter, prion cellular protein

INTRODUCTION

Air pollution is a significant health problem in megacities around the world (Molina and Molina, 2004; Chen and Kan, 2008; Bloom, 2011). In the first 50 years of this century (Bloom, 2011), the projected world population will have a further increase of 2–4.5 billion making the issue of deteriorating environments and their health impact critical.

Mexico City Metropolitan Area (MCMA) children with no known risk factors for neurological or cognitive disorders exhibit significant deficits in a combination of fluid and crystallized cognition tasks vs. low air pollution exposed children (Calderón-Garcidueñas et al., 2008a). Brain structural and volumetric changes are seen in both MCMA children and young animal facility dogs (Calderón-Garcidueñas et al., 2008a, 2011a, 2012a). The

canine frontal white matter lesions are characterized by vascular sub-cortical pathology associated with neuroinflammation, gliosis, and ultrafine particulate matter deposition (Calderón-Garcidueñas et al., 2008a). Young MCMA residents exhibit the neuropathological hallmarks of Alzheimer and Parkinson's diseases i.e., amyloid beta42 (Aβ42) plaques, tau hyperphosphorylation with pre-tangles and α-synuclein accumulation (Calderón-Garcidueñas et al., 2012b).

The complex modulation of cytokines and chemokines that influence children's central nervous system (CNS) structural and volumetric responses to air pollution obligate the search for biomarkers associating systemic and CNS inflammation to brain growth and cognitive deficits in the short term and neurodegeneration in the long-term (Calderón-Garcidueñas et al., 2008a,b, 2011a,b, 2012b). A good candidate biomarker, macrophage migration inhibitory factor (MIF) is a cytokine expressed in the CNS and involved in innate and adaptive immune responses (Bernhagen et al., 1993; Bacher et al., 2010; Edwards et al., 2010; Moon et al., 2012; Savaskan et al., 2012; Bucala, 2013; Cox et al., 2013; Freiria-Oliveira et al., 2013; Turtzo et al., 2013). MIF participates in the induction of neural stem/progenitor cells (Ohta et al., 2012), the protection of mice female brains after experimental stroke (Turtzo et al., 2013), the mediation of the antidepressant action of exercise (Moon et al., 2012), and of key importance for this work, MIF plays a key role in neuroinflammatory responses (Savaskan et al., 2012; Cox et al., 2013).

In clinical practice and in the experimental setting, MIF has been used as a marker of neurological worsening in progressive neurodegenerative processes including multiple sclerosis and autoimmune-mediated neuroinflammation (Hagman et al., 2011; Cox et al., 2013), as a key neuro-immune mediator linking depressive symptoms with inflammation and HPA dysregulation (Edwards et al., 2010), and as a predictor of lack of response to antidepressants (Cattaneo et al., 2013). Moreover, in the scenario of air pollution and the early hallmarks of Alzheimer's disease (AD) in Mexico City children (Calderón-Garcidueñas et al., 2008a, 2012a), two key issues are of utmost interest to us: the increased concentrations of MIF in the cerebro-spinal-fluid (CSF) of AD patients and the capacity of MIF to enhance the ability of the tau/Aβ42 ratio to discriminate cognitively normal vs. mildly demented patients (Lee et al., 2008; Bacher et al., 2010; Craig-Schapiro et al., 2011). In addition MIF promotes the production of several inflammatory mediators including IL-6, TNF α, and IFN γ (Popp et al., 2009). Given the wide spectrum of serum and CSF responses to pathological processes including neuroinflammation and neurodegenerative diseases such as AD and Parkinson's disease (PD) (Dziedzic, 2006; Helmy et al., 2012; Yan et al., 2012; Giralt et al., 2013; Martinez et al., 2013; Mooijaart et al., 2013; Smolen et al., 2013), we have selected a 41 cytokine/chemokine panel and MIF to explore the CSF responses to high air pollution residency.

Cellular prion protein (PrP^C) is a conserved GPI-anchored membrane protein located on the surface of neurons, at both pre and post-synaptic sites, with a high abundance in the hippocampus, frontal cortex and stratium (Stellato et al., 2011; Ding et al., 2013; Watt et al., 2013). The PRNP gene located in chromosome 20 likely plays key roles in neuronal development, synaptic plasticity, myelin sheath maintenance, cell adhesion, CNS development, and neuroprotection via inhibition of the mitochondrial apoptotic pathway (Mitsios et al., 2007; Altmeppen et al., 2012; Bradford and Mabbott, 2012; Kaiser et al., 2012; Ding et al., 2013).

We are particularly interested in the critical role PrP^C may play in the integrity of the CNS under a neuroinflammatory insult, precisely the key marker of air pollution exposure in urban children (Bremer et al., 2010; Popko, 2010; Gourdain et al., 2012; Scalabrino and Veber, 2012). The role of PrP^C in cellular immunity (Tsutsui et al., 2008), cognitive functioning (Breitling et al., 2012), oxidative stress, and the impact of prion proteins upon the toxic effect of environmental neurotoxic metals (Choi et al., 2007; Oh et al., 2012) are at the core of our research.

The primary aim of this study was to measure the concentrations of PrP^C and selected inflammatory mediators in normal CSF from cohorts of children with high exposures to urban air pollution vs. low pollution control children. Concurrently, we also explored serum MIF and PrP^C in clinically healthy children representing cohorts with high and low air pollution exposures.

Our results identify CSF as a MIF, IL6, IL-1Ra, IL-2, and PrP^C compartment that differentiates children with high vs. low air pollution exposures. The MIF increases in CSF and serum compartments in highly vs. low exposed children strongly suggest MIF could be a serum biomarker bridge to identify children at risk for brain inflammation. Short and long term effects of dysregulated neural immune responses and systemic inflammation are of pressing importance for public health.

PROCEDURE

STUDY AREAS

Children's cohorts were selected from the MCMA and several small cities in Mexico, characterized by clean environments with concentrations of the six criteria air pollutants (ozone, particulate matter, sulfur dioxide, nitrogen oxides, carbon monoxide and lead) below the current US EPA standards.

PARTICIPANTS

This research was approved by the research ethics committee of the Hospital Central Militar in Mexico City. Children gave active assent and their parents gave written informed consent to participation in the study. This work includes data from 139 children 74F, 65M (*Meanage* = 11.91 years, *SD* = 4.2), carefully selected to represent comparable populations from a larger longitudinal cohort research program. There were two groups of children included in this study: Group 1 (*n*: 28, *Meanage* = 10.46 years, *SD* = 4.2, low pollution 8F/6M; high pollution 6F/8M) corresponded to children admitted to the hospital from either MCMA or a low polluted city with a work up diagnosis of acute lymphoblastic leukemia entering a clinical protocol, which included a spinal tap. Only cases with a normal CSF were included in this study. None of the selected CSF samples belonged to children with previous oncological and/or hematological treatments. Group 2 (*n*: 111, 44 controls (24F/20M), 67 MCMA (33F/34M), *Meanage* = 13.37 years, *SD* = 4.2) were clinically healthy children from MCMA and control cities and their serum samples were taken as part of their pediatric examination during a longitudinal follow-up. Inclusion criteria for all participating children

were: negative smoking history and environmental tobacco exposure, lifelong residency in MCMA or a control city, residency within 5 miles of the city monitoring stations, full term birth, and unremarkable clinical histories prior to the hospital admission (for the children CSF donors). Low and high pollution exposed children were matched by age, gender, and socioeconomic status.

CSF SAMPLES

Spinal tap was performed in the supine position from lumbar levels using a standard 22 spinal needle. CSF was collected dripping in free air in1 ml aliquot into Nalge Nunc polypropylene CryoTubes. Lumbar puncture samples were collected during non-traumatic, non-complicated procedures. CSF pleocytosis was defined as CSF white blood cell (WBC) counts of >7 cells per mm^3. CSF samples were used for the laboratory procedures without dilution steps.

PERIPHERAL BLOOD ANALYSIS

Blood was collected from an antecubital vein using a 21-G needle. After centrifugation at 3000 rpm for 10 min, aliquots of 1.5 ml serum were transferred to CryoTubes and samples were frozen at −20°C and then transferred to −80°C and stored until further analysis. Blood samples were also collected for a complete blood count (CBC) with differential. Serum samples were processed for Multiplexing LASER Bead Technology (Eve Technologies, Calgary, Canada) that included 41 cytokines and chemokines: EGF, Eotaxin, FGF-2, Flt-3L, Fractalkine, G-CSF, GM-CSF, GRO, IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17a, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGFα, TNFα, TNFβ, and VEGF. An ELISA was done for the quantification of PrPc in serum and CSF, BetaPrion®Human EIA (AJ RoboScreen GmbH, Leipzig, Germany). Macrophage inhibitory factor (MIF) was done using an ELISA procedure from R&D (Minneapolis, MN 55413, USA). Serum samples were run without dilution steps.

DATA ANALYSIS

First, we calculated the summary statistics (mean ± standard deviation) of all relevant variables including age, inflammatory mediators and PrPc results. In the 28 CSF samples, we worked with 42 variables and established with independent samples *t*-tests which variables were statistically significant between children with high exposures to air pollutants (Mexico City) and children residing in low pollution cities (Controls). Spearman correlations were run between the CSF target inflammatory mediators and PrPc. In the 111 serum samples, we worked with 2 variables and established with independent samples *t*-tests which variables were statistically significant between Mexico City and control children. Significance was assumed at $p < 0.05$ and data expressed as mean values ± SD. All the statistical analyses described above were performed on statistical software "R" (http://www.r-project.org/).

RESULTS

CSF DATA

CSF samples were clear, colorless, with a normal opening pressure, a mean WBC count of 2.1 ± 1 cells per mm^3 and no RBC. Glucose was 55.9 ± 6.7 mg/100 ml in controls and 54.8 ± 11.7 mg/100ml in Mexico City children ($p = 0.3$). **Table 1** shows the results of the 42 variables included in the analysis. Six variables

Table 1 | Cerebro-spinal- fluid inflammatory mediators in Control vs. Mexico City children (values are shown in pg/ml).

Variables	Mean (controls)	*SD* (controls)	Mean (Mexico City)	*SD* (Mexico City)	*p*-value
EGF	1.627	2.258	2.416	2.665	0.396
FGF2	11.972	20.495	13.294	12.858	0.838
TGFalpha	2.810	1.637	1.800	1.667	0.111
GCSF	8.389	3.668	10.602	12.673	0.526
FIT.3L	23.429	18.261	35.249	34.856	0.261
GM.CSF	4.327	2.253	2.907	1.500	0.060
Fractalkine	41.711	18.529	218.851	422.998	0.127
IFNα2	4.931	2.468	5.349	2.989	0.683
IFNγ	0.278	0.148	1.296	2.006	0.070
GRO	8.057	13.421	19.084	22.972	0.125
IL10	0.259	0.258	2.593	4.966	0.091
MCP3	3.574	3.358	3.594	5.018	0.990
IL12p40	2.030	1.131	2.526	1.564	0.335
MDC	16.996	6.852	20.768	11.320	0.285
IL12	0.473	0.939	0.637	0.516	0.571
PDGF.AA	4.351	4.690	3.210	3.483	0.467
IL13	0.516	0.229	0.477	0.255	0.673
PDGF-BB	0.963	1.494	0.635	1.301	0.535
IL15	2.920	2.491	5.237	5.317	0.144
sCD40l	2.391	5.617	1.334	1.547	0.507
IL17a	5.165	18.595	0.225	0.021	0.338
IL1ra	0.475	0.579	1.915	1.954	0.014
IL1a	0.323	0.365	0.905	1.329	0.122
IL9	0.996	1.229	0.952	0.861	0.913
IL1beta	0.231	0.043	0.642	1.389	0.272
IL2	0.218	0.042	0.530	0.532	0.040
IL3	0.220	0	0.709	1.929	0.343
IL4	2.099	0.890	2.788	1.627	0.167
IL5	0.534	0.385	3.769	12.514	0.334
IL6	1.161	0.650	21.26	38.5	0.006
IL7	0.251	0.080	0.429	0.445	0.147
IL8	17.306	26.099	26.341	37.853	0.459
IP10	207.479	492.590	296.127	519.441	0.641
MCP1	1061.199	1159.217	1521.123	2698.525	0.553
MIP1α	1.130	1.846	6.497	9.129	0.041
MIP1β	6.776	3.065	7.049	3.761	0.832
RANTES	2.121	1.720	8.667	13.963	0.093
TNFα	0.368	0.224	1.441	3.0208	0.191
TNFβ	0.471	0.843	0.987	2.384	0.442
VEGF	4.542	4.494	24.503	43.563	0.099
PrPc	4.396	3.228	8.036	5.506	0.039
MIF	66.918	24.454	329.930	264.342	0.002

were statistically significant, including: MIF, PrP^C, IL-6, IL1Ra, IL-2, and MIP1α (this is not an effect of the number of multiple comparisons, i.e., inflation of Type I error, because the binomial probability that 6 comparisons out of 42 would turn out to be significant by chance is less than 0.0001). Relevant Spearman correlations between CSF PrP^C and target inflammatory mediators are seen in **Table 2**. We found significant correlations between PrP^C and IL-15 ($p = 0.025$) and RANTES ($p = 0.04$), MIF and IL-6 ($p < 0.000$) and IL-6 with IL-15 and RANTES ($p = 0.03$).

SERUM DATA

MIF and PrP^C results in serum are seen in **Table 3**. MIF was significantly higher ($p = 0.009$) in Mexico City children, while PrP^C showed no differences between cohorts.

DISCUSSION

Cerebro-spinal-fluid concentrations of Macrophage migration inhibitory factor MIF, IL-6, IL-1Ra, IL-2, Macrophage Inflammatory Protein 1-α [MIP1α,Chemokine (C-C Motif) Ligand 3] and PrP^C differentiate children with high vs. low air pollution exposures and suggest a complex interplay of a network of multipotent cytokines and normal cellular proteins with known neuromodulatory actions participating in neuroinflammatory responses associated with exposures to air pollutants. Our data also suggests that MIF in serum is a potential biomarker bridge to identify children with CNS inflammation.

IL-6, IL-1Ra, IL-2, and MIF are important players in the pathogenesis of neuroinflammation and the systemic inflammation associated with chronic exposures to high concentrations of air pollutants, while the increased CSF concentrations of PrP^C could potentially represent a neuroprotective response against oxidative stress (Griffiths et al., 2007; Carnini et al., 2010).

IL-6 is a pleiotropic cytokine with key roles in inflammatory responses and in directing T cell differentiation in adaptive immunity. The elevation of IL-6 in the CSF of highly exposed children and its significant correlation with other potent chemokines and cytokines regulating and coordinating innate and adaptive immune responses is important in the developing CNS.

IL-6 has been implicated in systemic and brain neoangiogenesis (Gertz et al., 2012). In traumatic brain injury the presence of low concentrations of IL-6 [IL-6 knock-out (KO) mice] is associated with poor behavior performance, and impacts the expression of IL1β, another powerful pro-inflammatory cytokine (Ley et al., 2011). Neuroprotective associations of specific IL-6 SNP (i.e., the G-allele of the SNP rs1800795) on hippocampal volumes (Baune et al., 2012), the protection of midbrain dopaminergic neurons by IL-6 in the model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Spittau et al., 2012), and the significant attenuation of IL-6 concentrations and lower gray volume in old rhesus macaques by a caloric-restricted diet (Willette et al., 2010) support a complex interaction between IL-6, other inflammatory mediators and protective brain responses. The role of IL-6 in long-term potentiation (LTP) and learning is well known (Del Rey et al., 2013). The increased expression of IL-6 is causally related to an increase in synaptic strength since it was abrogated when LTP was interfered by blockade of NMDA-glutamate receptors (Del Rey et al., 2013).

Detrimental effects on the other hand, are described between high concentrations of IL-6, reduced hippocampal volumes and major depressive disorders (Frodl et al., 2012) and in chronic systemic inflammatory conditions with increased risk of stroke (Drake et al., 2011). In advanced HIV infection, CSF concentrations of IL-6 show elevations in patients on suppressive combination antiretroviral therapy regardless of cognitive status, implying the persistence of intra-thecal inflammation even in the absence of clinical manifestations (Kamat et al., 2012).

Of utmost importance for this work and the higher concentrations of CSF IL-6 in Mexico City children is the fact we are seeing the hallmarks of Alzheimer i.e., hyperphosphorilated tau and Aβ42 amyloid plaques in 44 and 51%, respectively, of highly exposed Mexico City teens vs. 0% in low pollution control children. In the literature involving elderly individuals, the associations between IL-6 and dementia are controversial (Papassotiropoulos et al., 2001; Jellinger, 2010; Helmy et al., 2012). Galimberti et al., reported the highest CSF IL-6 concentrations in AD patients with mild cognitive deterioration, suggesting to the authors a key role of IL-6 in the initial phases of neurodegeneration (Galimberti et al., 2008). Combarros et al., suggested that dysregulation of IL-6 in some elderly people contributes to the development of AD (Combarros et al., 2009). Oxidative damage and neuroinflammation are crucial players in neurodegenerative diseases (Nunomura et al., 2001, 2006, 2012a,b), and both are present in the context of air pollution exposures (Calderón-Garcidueñas et al., 2012a). Moreover, under experimental conditions, exposures to different components of air pollutants

Table 3 | Serum MIF and PrP^C in Control v Mexico City children.

Variable	Controls	Mexico City	*p*-value
MIF	425.1 ± 188	711.1 ± 488.7	0.009
PrP^C	1.671 ± 0.96	1.647 ± 1.465	0.21

Values are shown in pg/ml.

Table 2 | Spearman correlations in CSF target inflammatory mediators and PrP^C Control v Mexico City children.

Mediator	PrP^C *r*/*p*-value	MIF *r*/*p*-value	IL-6 *r*/*p*-value	IL-15 *r*/*p*-value	RANTES *r*/*p*
PrP^C	1000	0.307 /0.113	0.236/0.302	0.424/0.025	0.406/0.044
MIF	0.307/0.113	1000	0.773/0.000	0.233/0.232	0.236/0.256
IL-6	0.236/0.302	0.773/0.000	1000	0.467/0.033	0.476/0.034
IL-15	0.424/0.025	0.233/0.232	0.467/0.033	1000	0.692/0.000
RANTES	0.406/0.044	0.236/0.256	0.476/0.034	0.692/0.000	1000

cause oxidative stress, neuroinflammation and neurodegeneration (Calderón-Garcidueñas et al., 2003, 2008a, 2012a,b; Levesque et al., 2011, 2013). Thus, the CSF and brain inflammatory imbalance observed in highly exposed children could represent an early physiological reaction to chronic environmental stress contributing later to the establishment of neurodegenerative processes with childhood clinical manifestations (Calderón-Garcidueñas et al., 2008b, 2011b, 2012b, 2013). Of critical importance are the portals of entry of air pollutants in the urban setting: (i) the nasal pathway through the olfactory, trigeminal nerves and accessory posterolateral nerve, (ii) the red blood cells and monocytes transporting ultrafine PM and then delivering the particles to distant organs including the brain, (iii) the direct access of PM organic and inorganic components to the systemic circulation through the alveolar-capillary interphase, (iv) the gastrointestinal and vagal pathways (Calderón-Garcidueñas et al., 2007; Block and Calderón-Garcidueñas, 2009; Dhuria et al., 2010; Bleier et al., 2012; Lucchini et al., 2012).

Thus, the association between IL-6 serum increases and deficits in verbal memory (Grassi-Oliveira et al., 2011) obligates us to explore the cognitive consequences of an inflammatory imbalance and cognitive impairment in highly exposed cohorts (Aung et al., 2011; Tsai et al., 2012; Uski et al., 2012; Wittkopp et al., 2013).

Intrathecal inflammation in these children is counterbalanced by the increased production of interleukin 1 receptor antagonist (IL-1Ra), a natural endogenous antagonist of IL-1, critical for a variety of brain effects (Arend, 2002; Gadek-Michalska and Bugajski, 2010; Akash et al., 2013; Peters et al., 2013). IL-1RA exhibits low uptake in the normal brain, with rapid metabolism, a short biological half-life (4–6 h), and excretion via the kidneys (Cawthorne et al., 2011). IL-1Ra higher CSF concentrations in highly exposed children, is a key observation since we have previously described a significant frontal up-regulation of IL-1β in Mexico City children (Calderón-Garcidueñas et al., 2008a). IL-1Ra has clear neuroprotective effects in experimental perinatal inflammation and hypoxic-ischemic injuries (Girard et al., 2012), reduces ischemic brain damage and inflammation in co-morbid rats (Pradillo et al., 2012), prevents postoperative cognitive decline and neuroinflammation in older rats (Barrientos et al., 2012), and plays a key anti-inflammatory role in AD (Rubio-Perez and Morillas-Ruiz, 2012). Interestingly, experimental endotoxemia in healthy male volunteers produces significant transient increases in IL-1Ra and decreases in mood, likely reflecting a compensatory strategy or a greater social cognitive processing as a function of sickness (Kullmann et al., 2013). We interpreted the CSF IL-1Ra increases as a neuroprotective response in Mexico City children.

On the other hand, the increases in CSF IL-2 are in keeping with the critical role of this pro-inflammatory cytokine in both effector T-cell development and FoxP3(+) CD4(+) Treg-cell homeostasis (Shameli et al., 2013). Of utmost interest in the context of air pollution is the role of IL-2 in regulating inflammation in an organ-specific manner through the migration and retention of CD4(+) T-cells (both Th1 and Th2) (Ju et al., 2012). Since IL-2 is also required for regulating the Th2 cytokine response during T-cell activation and participates in multifactorial autoimmune responses (Wang et al., 2009; Baine et al., 2013), the issue of autoimmunity to CNS components warrants full investigation in exposed children especially those with extensive white matter hyperintense lesions (Calderón-Garcidueñas et al., 2008b, 2012b).

Macrophage Inflammatory Protein 1-α [Chemokine (C-C Motif) Ligand 3/CCR3] CSF increases in Mexico City children relate to its well known role in inflammatory responses, angiogenesis and tissue remodeling through binding with receptors to other chemokines (i.e., CCR1, CCR4, CCR5) (Gaspar et al., 2013).

MIF is a newly arrived player in the air pollution scenario, is an active participant in innate and acquired immunity (Savaskan et al., 2012), its association with the production of several inflammatory mediators including IL-6, TNF-α, and IFN-γ, and the identification of CSF MIF as a biomarker in AD and mild cognitive impairment patients (MCI) (Popp et al., 2009; Craig-Schapiro et al., 2011) has a great impact in air pollution-related brain effects.

The role of MIF as a neuro-immune mediator linking inflammation with depressive symptoms and HPA dysregulation (Anisman and Hayley, 2012; Savaskan et al., 2012), and its concomitant increases in CSF and serum makes MIF a potential biomarker to identify children with systemic and CNS inflammation. A facet of MIF of importance for us is the potentiation with autoimmune-mediated neuroinflammation (Cox et al., 2013). Cox et al. demonstrated that MIF is essential for microglial activation and the production of IL-6, IL-1β, TNF-α, and inducible NO synthase in an experimental autoimmune encephalomyelitis model. Cox et al., work is important to us because the target cytokines described in their paper are up-regulated in brain tissues of highly exposed children, and the same cytokines exhibit higher serum concentrations when compared with low pollution exposed children (Calderón-Garcidueñas et al., 2008a,b, 2009, 2011a, 2012a,b).

The CSF increases of the normal cellular isoform of the prion protein (PrP^C) in exposed children deserve to be discussed under three important headings:

1. The PrP^C neuroprotective role related primarily to its antioxidant properties, its capacity as a radical scavenger, its key role in signal transduction and cell survival, neuronal zinc regulation, and its functional importance in the protection against oxidative stress and metal toxicity (Brown et al., 1997; Kuwahara et al., 1999; Viles et al., 1999; Milhavet and Lehmann, 2002; Hu et al., 2007; Westergard et al., 2007; Carnini et al., 2010; Martins et al., 2010; Bertuchi et al., 2012; Alfaidy et al., 2013; Watt et al., 2013).
2. The PrP^C role in animal behavior suggesting that the absence of the prion protein could result in altered neural processing (Massimino et al., 2013).
3. The associations of PrP^C with neuropathology apart from the conventional prion diseases (Jiménez-Huete et al., 1998; Ferrer et al., 2001; Voigtländer et al., 2001; Checler and Vincent, 2002; Aguzzi and Haass, 2003; Rezaie et al., 2005; Schwarze-Eicker et al., 2005; Ramljak et al., 2008). Of interest to us are the controversial issues in the literature linking Aβ oligomers and

PrP^C (Laurén et al., 2009; Nygaard and Strittmatter, 2009; Gunther and Striitmatter, 2010; Kessels et al., 2010; Barry et al., 2011; Saijo et al., 2011; Larson et al., 2012; Um et al., 2012; Chen et al., 2013; Kudo et al., 2013; Whitehouse et al., 2013; Younan et al., 2013). A few specific examples to make the point: Kudo et al. showed that Prnp (-/-) mice are resistant to the neurotoxic effect of oligomeric Aβ *in vivo* and *in vitro* (Kudo et al., 2013). Chen et al. have shown PrP^C overexpression down-regulated tau protein and PrP^C lacking the Aβ oligomer binding site was incapable of rescuing the level of tau reduction. These authors demonstrated that PrP^C down-regulated tau via Fyn pathway and the effect can be regulated by Aβ oligomers (Chen et al., 2013). Whitehouse et al. measured PrP^C in the frontal cortex of 24 sporadic AD brains vs. 24 age-matched controls and found a significant decreased of PrP^C in AD brains. Interestingly, PrP^C significantly inversely correlated with BACE1 activity, Aβ load, soluble Aβ, and insoluble Aβ and with the stage of disease, as indicated by Braak tangle stage (Whitehouse et al., 2013). The authors concluded that brain PrP^C level may be important in influencing the onset and progression of sporadic AD (Whitehouse et al., 2013).

Why are all these papers relevant to our work? We recently published a paper (Calderón-Garcidueñas et al., 2012a) showing a 15 fold frontal down-regulation of mRNA PrP^C in Mexico City residents (18 ± 8.7 years) vs. low air pollution age matched controls. In the same work, we showed frontal tau hyperphosphorylation and Aβ diffuse plaques in the exposed kids, but none in low air pollution controls. Thus, the regional frontal PrP^C down-regulation shown by microarray analysis is of deep concern given the age of the autopsy subjects (on average 8 years older) than our CSF children in this study. PrP^C might have significant longitudinal variations in mRNA expression by brain region (Rezaie et al., 2005; Velayos et al., 2009), age, gender, and other factors in the setting of air pollution. Moreover, because the CSF is produced and reabsorbed throughout the entire CSF-Interstitial fluid (IF) functional unit (Chikly and Quaghebeur, 2013), our results highlight the need to define PrP^C changes both directly in different regions of the brain and in the CSF in low vs. high pollution exposed children and young adults. Furthermore, since PrP^C likely plays a key protective role against sporadic AD (Whitehouse et al., 2010; Griffiths et al., 2012) we will expect age-related decreases in specific regional brain areas inversely related to the development of AD hallmarks in highly exposed individuals.

The second reason for concern is the impact of PrP^C in cognition. Gimbel et al. showed that AD transgenic mice with intact PrP^C expression exhibit deficits in spatial learning and memory, while mice lacking PrP^C, but containing Aβ plaque derived from APPswe/PSen1DeltaE9 transgenes, show no detectable impairment of spatial learning and memory (Gimbel et al., 2010). Thus, deletion of PrP^C expression dissociates Aβ accumulation from behavioral impairment in AD mice, with the cognitive deficits selectively requiring PrP^C (Gimbel et al., 2010). Interestingly, Schmidt et al. (2013) showed no association between CSF PrP^C and cognitive status in 114 AD patients, while Breitling et al. reported in a 5 years follow-up study of 1322 elderly Germans (aged 65+ years at baseline), an inverse association between serum PrPc and cognitive functioning (Breitling et al., 2012). In our study, CSF PrP^C was significantly higher in exposed vs. low pollution children, while serum PrP^C concentrations showed no differences.

The significant correlations of PrP^C with RANTES and IL-15 deserve a comment. Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) is a proinflammatory chemokine produced by neurons and capable of upregulating neurotrophic factors and extending cell survival after ischemic stroke (Tokami et al., 2013), while IL-15 instructs the generation of a distinct memory T-lymphocyte subset, intermediate between naive and central memory cells (Cieri et al., 2013). These findings highlight the complex link between normal brain proteins, powerful chemokines, and immune driven cytokines.

It has been clear for a number of years that neuroinflammation has critical implications for cognition, behavior, altered brain growth and neurodegeneration. Brain dysbalances in inflammatory mediators and alterations of essential proteins with key roles in CNS development and neuroprotection will inevitably have detrimental consequences for young brains. We already described some of these consequences in Mexico City youth: differences in white matter volumes involving right parietal and bilateral temporal areas and cognitive deficits consistent with impairment of the targeted lobes, olfaction deficits, auditory and vestibular nuclei accumulating α synuclein and/or β amyloid and significant involvement of the medial superior olive neurons, critically involved in brainstem auditory evoked potentials. Elevation of indices of neuroinflammation and oxidative stress, and AD and PD associated pathology completing the grim picture (Calderón-Garcidueñas et al., 2003, 2008a,b, 2010, 2011a,b, 2012a,b, 2013; Calderón-Garcidueñas and Torres-Jardón, 2012).

At the core of our observations lies the complex dynamic interaction in different compartments of cytokines, chemokines and brain proteins responding to the constant state of inflammation and oxidative stress resulting from unhealthy environmental exposures. The short term consequences are having an impact in the performance of children in school and in their violent behavior (Calderón-Garcidueñas and Torres-Jardón, 2012), while the longer effects are already seen in neuropathological studies of children with accidental deaths (Calderón-Garcidueñas et al., 2008b, 2012b, 2013). There is no doubt of the close linkage between the brain and the immune system particularly in the developing brain (Bilbo and Schwarz, 2012). Brisk responses are at hand both systemically and in the brain upon endogenous and exogenous environmental signals, however, the developing brain is delicately sensitive to chronic stimuli and the end result could be a pathological process with deleterious outcomes for the individual.

LOOKING FORWARD AND LIMITATIONS

Despite controversy regarding the mechanistic pathways involved in the CNS damage associated with exposure to air pollutants, we agree that oxidative stress, endothelial dysfunction vascular damage and neuroinflammation are at the core of the pathology,

and the same factors play a key role in neurodegenerative diseases (Qin et al., 2007; Rivest, 2009; Levesque et al., 2011; Castellani and Perry, 2012; de la Torre, 2012; Nunomura et al., 2012a,b; Calderón-Garcidueñas et al., 2013). We are looking forward to bridging the gap between early neuroinflammation and its neurodegenerative consequences, an issue of importance in childhood and adolescence when cognitive performance is critical.

There is a need for looking into the CSF early responses to air pollution because we can connect with the knowledge available for elderly populations, i.e., the progression of Alzheimer core biomarkers [Aβ(1-42), total tau and phosphorylated tau] and inflammatory markers useful in the clinical practice to evaluate patients with mild cognitive impairment and their course to AD. This is critical, given that implementation of early preventive pathways (Castellani and Perry, 2012) in the developing brain could ameliorate or drastically modified the pathology in later years.

Our results are potentially limited by the fact our CSF normal samples were taken from a population of children with a hematological workup for a neoplastic process, albeit no CNS involvement, while our serum samples were from clinically healthy children. As pediatricians we are totally aware of the ethical issues in taking CSF samples from healthy children, so the readily available normal CSF samples destined to be discarded are a good initial source of exploratory samples. Nevertheless, the robust CSF increase in the described inflammatory mediators in highly exposed children warrants extensive investigations using available normal CSF samples in Mexico and around the world. Two issues are important here: the selection of multiplex platforms and knowledge of their ability to accurately and sensitively detect cytokines in CSF and blood (serum/plasma) (Malekzadeh et al., 2012). The second one, the use of standardized operating procedures for the recollection of CSF samples, a key point if we want to decrease the variability attributed to different preanalytical procedures between laboratories (del Campo et al., 2012).

SUMMARY

We have quantified CSF responses for a selected panel de inflammatory mediators and PrP^C in two groups of children with contrasting air pollution lifetime exposures. Our results identify MIF, IL6, IL1Ra, IL-2, and PrP^C as potential CSF biomarkers differentiating the high vs. low air pollution exposed children, supporting the previous evidence of an ongoing neuroinflammatory process in Mexico City children. Serum MIF was identified as a robust biomarker in Mexico City children.

Fine tuning of immune-to-brain communication is crucial to neural networks appropriate functioning, thus the short and long term effects of systemic inflammation and dysregulated neural immune responses are of deep concern for millions of highly exposed children.

A large body of work on PrP^c, IL-6, MIF, IL-2, and the IL-1 family already exists, thus expanding this knowledge in the scenario of air pollution pediatric effects could greatly facilitate our understanding of the downstream mechanisms of the complex interaction of cytokines/chemokines/ PrP^c immune responses in the developing brain and the resulting short and long term brain effects.

The role of PrP^c in highly exposed children is an issue worth investigating further as the field moves forward, since we believe knowledge on PrP^c responses in a developing brain will shed light on neuroprotective mechanisms against the onset of Alzheimer disease.

Although the two objectives of this study were accomplished: (i) To evaluate the concentrations of PrP^C and selected inflammatory mediators in normal CSF from cohorts of children with high vs. low pollution controls and (ii) To explore serum PrP^C and MIF in clinically healthy children representing cohorts with high and low air pollution exposures, our immediate goal will be to assess whether the identified biomarkers will be useful in the identification of children at higher risk for cognitive deficits, behavioral disorders, and structural and volumetric brain alterations.

Our ultimate goal is that following the identification of children at the highest risk for detrimental brain effects, we protect them through multidimensional interventions yielding both impact and reach. The prospective of our efforts is readily apparent.

Defining the linkage and the health consequences of the brain/immune system interactions in the developing brain chronically exposed to air pollutants ought to be of pressing importance for public health.

AUTHOR CONTRIBUTIONS

Lilian Calderón-Garcidueñas planned and directed the study, wrote the manuscript, performed all experiments and supported the study from personal funds. Janet V. Cross planned the study, performed the MIF determinations and wrote the manuscript. Amedeo D'Angiulli wrote, edited and formatted the manuscript and performed part of the statistic analysis. Maricela Franco-Lira and Mariana Aragón-Flores obtained patient consent and ethical approvals, got the samples and wrote the manuscript. Michael Kavanaugh wrote the manuscript. Chih-kai Chao and Charles Thompson performed the PrP^C and wrote the manuscript. Ricardo Torres-Jardón wrote the manuscript and Jing Chang and Hongtu Zhu performed the statistical analysis. All authors approved the final draft of the manuscript for publication.

ACKNOWLEDGMENTS

This work was supported in part by the UM-CHPBS Endowment Fund Program, NIMHD grant # S21-MD000236 and a AUCC LACREG grant to Drs. Calderón and D'Angiulli. We thank Dennis Templeton, MD PhD, Chair, Department of Pathology, School of Medicine, University of Virginia, Charlottesville, VA for his generous support of this research while Dr. Lilian Calderón-Garcidueñas was a U Virginia visiting professor. We also thank Lara Ferreira-Azevedo, 5th year Pharmacy visiting student from Brazil for her technical assistance.

REFERENCES

Aguzzi, A., and Haass, C. (2003). Games played by rogue proteins in prion disorders and Alzheimer's disease. *Science* 302, 814–818. doi: 10.1126/science.1087348

Akash, M. S., Rehman, K., and Chen, S. (2013). IL-1Ra and its delivery strategies: inserting the association in perspective. *Pharm. Res.* doi: 10.1007/s11095-013-1118-0. [Epub ahead of print].

Alfaidy, N., Chauvet, S., Donadio-Andrei, S., Salomon, A., Saoudi, Y., Richaud, P., et al. (2013). Prion protein expression and functional importance in developmental angiogenesis: role in oxidative stress and copper homeostasis. *Antioxid. Redox Signal.* 18, 400–411. doi: 10.1089/ars.2012.4637

Altmeppen, H. C., Puig, B., Dohler, F., Thurm, D. K., Falker, C., Krasemann, S., et al. (2012). Proteolytic processing of the prion protein in health and disease. *Am. J. Neurodegener. Dis.* 1, 15–31.

Anisman, H., and Hayley, S. (2012). Inflammatory factors contribute to depression and its comorbid conditions. *Sci. Signal.* 5:pe45. doi: 10.1126/scisignal.2003579

Arend, W. P. (2002). The balance between IL-1 and IL-1Ra in disease. *Cytokine Growth Factor Rev.* 13, 323–340. doi: 10.1016/S1359-6101(02)00020-5

Aung, H. H., Lame, M. W., Gohil, K., He, G., Denison, M. S., Rutledge, J. C., et al. (2011). Comparative gene responses to collected ambient particles *in vitro*: endothelial responses. *Physiol. Genomics* 43, 917–929. doi: 10.1152/physiolgenomics.00051.2011

Bacher, M., Deuster, O., Aljabari, B., Egensperger, R., Neff, F., Jessen, F., et al. (2010). The role of macrophage migration inhibitory factor in Alzheimer's disease. *Mol. Med.* 16, 116–121. doi: 10.2119/molmed.2009.00123

Baine, I., Basu, S., Ames, R., Sellers, R. S., and Macian, F. (2013). Helios induces epigenetic silencing of IL2 gene expression in regulatory T cells. *J. Immunol.* 190, 1008–1016. doi: 10.4049/jimmunol.1200792

Barrientos, R. M., Hein, A. M., Frank, M. G., Watkins, L. R., and Maier, S. F. (2012). Intracisternal interleukin-1 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory response in aged rats. *J. Neurosci.* 32, 14641–14648. doi: 10.1523/JNEUROSCI.2173-12.2012

Barry, A. E., Klyubin, I., McDonald, J. M., Mably, A. J., Farrell, M. A., Scott, M., et al. (2011). Alzheimer's disease brain-derived amyloid-β-mediated inhibition of LTP *in vivo* is prevented by immunotargeting cellular prion protein. *J. Neurosci.* 31, 7259–7263. doi: 10.1523/JNEUROSCI.6500-10.2011

Baune, B. T., Konrad, C., Grotegerd, D., Suslow, T., Birosova, E., Ohrmann, P., et al. (2012). Interleukin-6 gene (IL6): a possible role of brain morphology in the healthy adult brain. *J. Neuroinflammation* 9:125. doi: 10.1186/1742-2094-9-125

Bernhagen, J., Calandra, T., Mitchell, R. A., Martin, S. B., Tracey, K. J., Voelter, W., et al. (1993). MIF is a pituitary-derived cytokine that potentiates lethal endotoxemia. *Nature* 365, 756–759. doi: 10.1038/365756a0

Bertuchi, F. R., Bourgeon, D. M., Landemberger, M. C., Martins, V. R., and Cerchiaro, G. (2012). PrPC displays an essential protective role from oxidative stress in an astrocyte cell line derived from PrPC knockout mice. *Biochim. Biophys. Res. Commun.* 418, 27–32. doi: 10.1016/j.bbrc.2011.12.098

Bilbo, S. D., and Schwarz, J. M. (2012). The immune system and developmental programming of brain and behavior. *Front. Neuroendocrinol.* 33, 267–286. doi: 10.1016/j.yfrne.2012.08.006

Bleier, B. S., Feldman, R., Sadow, P. M., Wu, A., Ting, J., and Metson, R. (2012). The accessory posterolateral nerve: an immunohistological analysis. *Am. J. Rhinol. Allergy* 26, 271–273. doi: 10.2500/ajra.2012.26.3804

Block, M., and Calderón-Garcidueñas, L. (2009). Air Pollution: mechanisms of neuroinflammation and CNS disease. *Trends Neurosci.* 32, 506–516. doi: 10.1016/j.tins.2009.05.009

Bloom, D. E. (2011). 7 Billion and counting. *Science* 333, 562–569. doi: 10.1126/science.1209290

Bradford, B. M., and Mabbott, N. A. (2012). Prion disease and the innate immune system. *Viruses* 4, 3389–3419. doi: 10.3390/v4123389

Breitling, L. P., Muller, H., Stegmaier, C., Kliegel, M., and Brenner, H. (2012). Association of prion protein with cognitive functioning in humans. *Exp. Gerontol.* 47, 919–24. doi: 10.1016/j.exger.2012.08.001

Bremer, J., Baumann, F., Tiberi, C., Wessig, C., Fischer, H., Schwarz, P., et al. (2010). Axonal prion protein is required for peripheral myelin maintenance. *Nat. Neurosci.* 13, 310–318. doi: 10.1038/nn.2483

Brown, D. R., Schmidt, B., and Kretzschmar, H. A. (1997). Effects of oxidative stress on prion protein expression in PC12 cells. *Int. J. Dev. Neurosci.* 15, 961–972. doi: 10.1016/S0736-5748(97)00042-7

Bucala, R. (2013). MIF, MIF alleles, and prospects for therapeutic intervention in autoimmunity. *J. Clin. Immunol.* 33(Suppl. 1), S72–S78. doi: 10.1007/s10875-012-9781-1

Calderón-Garcidueñas, L., Engle, R., Mora-Tiscareño, A., Styner, M., Gomez-Garza, G., Zhu, H., et al. (2011a). Exposure to severe urban pollution influences cognitive outcomes, brain volume and systemic inflammation in clinically healthy children. *Brain Cognition* 77, 345–355. doi: 10.1016/j.bandc.2011.09.006

Calderón-Garcidueñas, L., D'Angiulli, A., Kulesza, R. J., Torres-Jardón, R., Romero, L., Keefe, S., et al. (2011b). Air pollution is associated with brainstem auditory nuclei pathology and delayed brainstem auditory evoked potentials. *Int. J. Dev. Neurosci.* 29, 365–375. doi: 10.1016/j.ijdevneu.2011.03.007

Calderón-Garcidueñas, L., Franco-Lira, M., Henríquez-Roldán, C., González-Maciel, A., Reynoso-Robles, R., Villarreal-Calderon, R., et al. (2010). Urban air pollution: influences on olfactory function and pathology in exposed children and young adults. *Exp. Toxicol. Pathol.* 62, 91–102. doi: 10.1016/j.etp.2009.02.117

Calderón-Garcidueñas, L., Franco-Lira, M., Mora-Tiscareño, A., Medina-Cortina, H., Torres-Jardón, R., and Kavanaugh, M. (2013). Early Alzheimer's and Parkinson's disease pathology in urban children: friend versus foe responses-it is time to face the evidence. *Biomed. Res. Int.* doi: 10.1155/2013/161687

Calderón-Garcidueñas, L., Franco-Lira, M., Torres-Jardón, R., Henriquez-Roldán, C., Valencia-Salazar, G., González-Maciel, A., et al. (2007). Pediatric and systemic effects of chronic air pollution exposure: nose, lung, heart, and brain pathology. *Toxicol. Pathol.* 35, 154–162. doi: 10.1080/01926230601059985

Calderón-Garcidueñas, L., Kavanaugh, M., Block, M. L., D'Angiulli, A., Delgado-Chávez, R., Torres-Jardón, R., et al. (2012a). Neuroinflammation, hyperphosphorilated tau, diffuse amyloid plaques and down- regulation of the cellular prion protein in air pollution exposed children and adults. *J. Alzheimers Dis.* 28, 93–107.

Calderón-Garcidueñas, L., Mora-Tiscareño, A., Styner, M., Gómez-Garza, G., Zhu, H., Torres-Jardón, R., et al. (2012b). White matter hyperintensities, systemic inflammation, brain growth and cognitive functions in children exposed to air pollution. *J. Alzheimers Dis.* 31, 183–91.

Calderón-Garcidueñas, L., Macías-Parra, M., Hoffmann, H. J., Valencia-Salazar, G., Henríquez-Roldán, C., Monte, O. C., et al. (2009). Immunotoxicity and environment: immunodysregulation and systemic inflammation in children. *Toxicol. Pathol.* 37, 161–169. doi: 10.1177/0192623308329340

Calderón-Garcidueñas, L., Maronpot, R. R., Torres-Jardón, R., Henriquez-Roldán, C., Schoonhoven, R., Acuña-Ayala, H., et al. (2003). DNA damage in nasal and brain tissues of canines exposed to air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration. *Toxicol. Pathol.* 31, 524–538. doi: 10.1080/01926230390226645

Calderón-Garcidueñas, L., Solt, A. C., Henríquez-Roldán, C., Torres-Jardón, R., Nuse, B., Herritt, L., et al. (2008a). Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood-brain-barrier, ultrafine particulate deposition, and accumulation of amyloid beta-42 and alpha-synuclein in children and young adults. *Toxicol. Pathol.* 36, 289–310.

Calderón-Garcidueñas, L., Mora-Tiscareño, A., Gómez-Garza, G., Broadway, J., Chapman, S., Valencia-Salazar, G., et al. (2008b). Air pollution, cognitive deficits and brain abnormalities: a pilot study with children and dogs. *Brain Cogn.* 68, 117–127. doi: 10.1016/j.bandc.2008.04.008

Calderón-Garcidueñas, L., and Torres-Jardón, R. (2012). Air pollution, socioeconomic status and children's cognition in megacities: the Mexico City scenario. *Front. Psychol.* 3:217. doi: 10.3389/fpsyg.2012.00217

Carnini, A., Casha, S., Yong, V. W., Hurlbert, R. J., and Braun, J. E. (2010). Reduction of PrPC in human cerebrospinal fluid after spinal cord injury. *Prion* 4, 80–86. doi: 10.4161/pri.4.2.11756

Castellani, R. J., and Perry, G. (2012). Pathogenesis and disease-modifying therapy in Alzheimer's disease: the flat line of progress. *Arch. Med. Res.* 43, 694–698. doi: 10.1016/j.arcmed.2012.09.009

Cattaneo, A., Gennarelli, M., Uher, R., Breen, G., Farmer, A., Aitchison, K. J., et al. (2013). Candidate genes expression profile aassociated with antidepressants response in the GENDEP study: differentiating between baseline predictors and longitudinal targets. *Neuropsychopharmacology* 38, 377–385. doi: 10.1038/npp.2012.191

Cawthorne, C., Prenant, C., Smigova, A., Julvan, P., Maroy, R., Herholz, K., et al. (2011). Biodistribution, pharmacokinetics and metabolism of interleukin-1 receptor antagonist (IL-1RA) using [^{18}F] IL1RA and PET imaging in rats. *Br. J. Pharmacol.* 162, 659–672. doi: 10.1111/j.1476-5381.2010.01068.x

Checler, F., and Vincent, B. (2002). Alzheimer's and prion diseases: distinct pathologies, common proteolytic denominators. *Trends Neurosci.* 25, 616–620. doi: 10.1016/S0166-2236(02)02263-4

Chen, B., and Kan, H. (2008). Air pollution and population health: a global challenge. *Environ. Health Prev. Med.* 13, 94–101. doi: 10.1007/s12199-007-0018-5

Chen, R. J., Chang, W. W., Lin, Y. C., Cheng, P. L., and Chen, Y. R. (2013). Alzheimer's amyloid-β oligomers rescue cellular prion protein induced Tau reduction via Fyn pathways. *ACS Chem. Neurosci.* 4, 1287–1296. doi: 10.1021/cn400085q

Chikly, B., and Quaghebeur, J. (2013). Reassessing cerebrospinal fluid (CSF) hydrodynamics: a literature review presenting a novel hypothesis for CSF physiology. *J. Bodyw. Mov. Ther.* 17, 344–354. doi: 10.1016/j.jbmt.2013.02.002

Choi, C. J., Anantharam, V., Saetveit, N. J., Houk, R. S., Kanthasamy, A., and Kanthasamy, A. G. (2007). Normal cellular prion protein protects against manganese-induced oxidative stress and apoptotic cell death. *Toxicol. Sci.* 98, 495–509. doi: 10.1093/toxsci/kfm099

Cieri, N., Camisa, B., Cocchiarella, F., Forcato, M., Oliveira, G., Provasi, E., et al. (2013). IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood* 121, 573–584. doi: 10.1182/blood-2012-05-431718

Combarros, O., van Duijn, C. M., Hammond, N., Belbin, O., Arias-Vásquez, A., Cortina-Borja, M., et al. (2009). Replication by the epistasis project of the interaction between the genes for IL-6 and IL-10 in the risk of Alzheimer's disease. *J. Neuroinflammation* 6:22. doi: 10.1186/1742-2094-6-22

Cox, G. M., Kithcart, A. P., Pitt, D., Guan, Z., Alexander, J., Williams, J. L., et al. (2013). Macrophage migration inhibitory factor potentiates autoimmune-mediated neuroinflammation. *J. Immunol.* 191, 1043–1054. doi: 10.4049/jimmunol.1200485

Craig-Schapiro, R., Kuhn, M., Xiong, C., Pickering, E. H., Liu, J., Misko, T. P., et al. (2011). Multiplex immunoassay panel identifies novel CSF biomarkers for Alzheimer's disease diagnosis and prognosis. *PLoS ONE* 6:e18850. doi: 10.1371/journal.pone.0018850

de la Torre, J. C. (2012). A turning point for Alzheimer's disease? *Biofactors* 38, 78–83. doi: 10.1002/biof.200

del Campo, M., Mollenhauer, B., Bertolotto, A., Engelborghs, S., Hampel, H., Simonsen, A. H., et al. (2012). Recommendations to standardize preanalytical confounding factors in Alzheimer's and Parkinson's disease cerebrospinal fluid biomarkers: an update. *Biomark. Med.* 6, 419–430. doi: 10.2217/bmm.12.46

Del Rey, A., Balschun, D., Wetzel, W., Randolf, A., and Besedovsky, H. O. (2013). A cytokine network involving brain-borne IL-1β, IL-1Ra, IL-18, IL-6 and TNF-α operates during long-term potentiation and learning. *Brain Behav. Immun.* 33, 15–23. doi: 10.1016/j.bbi.2013.05.011

Dhuria, S. V., Hanson, L. R., and Frey, W. H. 2nd. (2010). Intranasal delivery to the central nervous system: mechanisms and experimental considerations. *J. Pharm. Sci.* 99, 1654–1673. doi: 10.1002/jps.21924

Ding, T., Zhou, X., Kouadir, M., Shi, F., Yang, Y., Liu, J., et al. (2013). Cellular prion protein participates in the regulation of inflammatory response and apoptosis in BV2 microglia during infection with Mycobacterium bovis. *J. Mol. Neurosci.* 51, 118–126. doi: 10.1007/s12031-013-9962-2

Drake, C., Boutin, H., Jones, M. S., Denes, A., McColl, B. W., Selvarajah, J. R., et al. (2011). Brain inflammation is induced by co-morbidities and risk factors for stroke. *Brain Behav. Immun.* 25, 1113–1122. doi: 10.1016/j.bbi.2011.02.008

Dziedzic, T. (2006). Systemic inflammatory markers and risk of dementia. *Am. J. Alzheimers Dis. Other Demen.* 21, 258–262. doi: 10.1177/1533317506289260

Edwards, K. M., Bosch, J. A., Engeland, C. G., Cacioppo, J. T., and Marucha, P. T. (2010). Elevated macrophage migration inhibitory factor (MIF) is associated with depressive symptoms, blunted cortisol reactivity to acute stress, and lowered morning cortisol. *Brain Behav. Immun.* 24, 1202–1208. doi: 10.1016/j.bbi.2010.03.011

Ferrer, I., Blanco, R., Carmona, M., Puig, B., Ribera, R., Rey, M. J., et al. (2001). Prion protein expression in senile plaques in Alzheimer's disease. *Acta Neuropathol.* 101, 49–56.

Freiria-Oliveira, A. H., Blanch, G. T., Li, H., Colombari, E., Colombari, D. S., and Sumners, C. (2013). Macrophage inhibitory factor in the nucleus of solitary tract decreases blood pressure in SHRs. *Cardiovasc. Res.* 97, 153–160. doi: 10.1093/cvr/cvs297

Frodl, T., Carballedo, A., Hughes, M. M., Saleh, K., Fagan, A., Skokauskas, N., et al. (2012). Reduced expression of glucocorticoid-inducible genes GILZ and SGK-1: high IL6 levels are associated with reduced hippocampal volumes in major depressive disorder. *Transl. Psychiatry* 2:e88. doi: 10.1038/tp.2012.14

Gadek-Michalska, A., and Bugajski, J. (2010). Interleukin-1 (IL-1) in stress-induced activation of limbic-hypothalamic-pituitary adrenal axis. *Pharmacol. Rep.* 62, 969–982.

Galimberti, D., Venturelli, E., Fenoglio, C., Guidi, I., Villa, C., Bergamaschini, L., et al. (2008). Intratechal levels of IL6, IL11 and LIF in Alzheimer's disease and frontotemporal lobar degeneration. *J. Neurol.* 255, 539–544. doi: 10.1007/s00415-008-0737-6

Gaspar, K., Kukova, G., Bunemann, E., Buhren, B. A., Sonkolv, E., Szollosi, A. G., et al. (2013). The chemokine receptor CCR3 participates in tissue remodeling during atopic skin inflammation. *J. Dermatol. Sci.* 71, 12–21. doi: 10.1016/j.jdermsci.2013.04.011

Gertz, K., Kronenberg, G., Kälin, R. E., Baldinger, T., Werner, C., Balkaya, M., et al. (2012). Essential role of interleukin-6 in post-stroke angiogenesis. *Brain* 135, 1964–1980. doi: 10.1093/brain/aws075

Gimbel, D. A., Nygaard, H. B., Coffey, E. E., Gunther, E. C., Laurén, J., Gimbel, Z. A., et al. (2010). Memory impairment in transgenic Alzheimer mice requires cellular prion protein. *J. Neurosci.* 30, 6367–6374. doi: 10.1523/JNEUROSCI.0395-10.2010

Giralt, M., Ramos, R., Quintana, A., Ferrer, B., Erta, M., Castro-Freire, M., et al. (2013). Induction of atypical EAE mediated by transgenic production of IL6 in astrocytes in the absence of systemic IL6. *Glia* 61, 587–600. doi: 10.1002/glia.22457

Girard, S., Sébire, H., Brochu, M. E., Briota, S., Sarret, P., and Sébire, G. (2012). Postnatal administration of IL-1Ra exerts neuroprotective effects following perinatal inflammation and/or hypoxic-ischemic injuries. *Brain Behav. Immun.* 26, 1331–1339. doi: 10.1016/j.bbi.2012.09.001

Gourdain, P., Ballerini, C., Nicot, A. B., and Carnaud, C. (2012). Exacerbation of experimental autoimmune encephalomyelitis in prion protein PrPc-null mice: evidence for a critical role of the central nervous system. *J. Neuroinflammation* 9:25. doi: 10.1186/1742-2094-9-25

Grassi-Oliveira, R., Bauer, M. E., Pezzi, J. C., Teixeira, A. L., and Brietzke, E. (2011). Interleukin-6 and verbal memory in recurrent major depressive disorder. *Neuro Endocrinol. Lett.* 32, 540–544.

Griffiths, H. H., Whitehouse, I. J., and Hooper, N. M. (2012). Regulation of amyloid-β production by the prion protein. *Prion* 6, 217–222. doi: 10.4161/pri.18988

Griffiths, M., Neal, J. W., and Gasque, P. (2007). Innate immunity and protective neuroinflammation: new emphasis on the role of neuroimmune regulatory proteins. *Int. Rev. Neurobiol.* 82, 29–55. doi: 10.1016/S0074-7742(07)82002-2

Gunther, E. C., and Striitmatter, S. M. (2010). Beta-amyloid oligomers and cellular prion protein in Alzheimer's disease. *J. Mol. Med.* 88, 331–338. doi: 10.1007/s00109-009-0568-7

Hagman, S., Raunio, M., Rossi, M., Dastidar, P., and Elovaara, I. (2011). Disease-associated inflammatory biomarker profiles in blood in different subtypes of multiple sclerosis: prospective clinical and MRI follow-up Study. *J. Neuroimmunol.* 234, 141–147. doi: 10.1016/j.jneuroim.2011.02.009

Helmy, A. A., Naseer, M. M., Shafie, S. E., and Nada, M. A. (2012). Role of interleukin 6 and alpha-globulins in differentiating Alzheimer and vascular dementias. *Neurodegener. Dis.* 9, 81–86. doi: 10.1159/000329568

Hu, W., Rosenberg, R. N., and Stüve, O. (2007). Prion proteins: a biological role beyond prion diseases. *Acta Neurol. Scand.* 116, 75–82. doi: 10.1111/j.1600-0404.2007.00868.x

Jellinger, A. K. (2010). Intrathecal levels of IL6 in Alzheimer's disease. *J. Neurol.* 257, 142. doi: 10.1007/s00415-009-5312-2

Jiménez-Huete, A., Lievens, P. M., Vidal, R., Piccardo, P., Ghetti,

B., Tagliavini, F., et al. (1998). Endogenous proteolytic cleavage of normal and disease-associated isoforms of the human prion protein in neural and non-neural tissues. *Am. J. Pathol.* 153, 1561–1572. doi: 10.1016/S0002-9440(10)65744-6

Ju, S. T., Sharma, R., Gaskin, F., and Fu, S. M. (2012). IL-2 controls trafficking receptor gene expression and Th2 response for skin and lung inflammation. *Clin. Immunol.* 145, 82–88. doi: 10.1016/j.clim.2012.07.015

Kaiser, D. M., Acharya, M., Leighton, P. L. A., Wang, H., Daude, N., Wohlgemuth, S., et al. (2012). Amyloid beta precursor protein and prion protein have a conserved interaction affecting cell adhesion and CNS development. *PLoS ONE* 7:e51305. doi: 10.1371/journal.pone.0051305

Kamat, A., Lyons, J. L., Misra, V., Uno, H., Morgello, S., Singer, E. J., et al. (2012). Monocyte activation markers in cerebrospinal fluid associated with impaired neurocognitive testing in advanced HIV infection. *J. Acquir. Immune Defic. Syndr.* 60, 234–243. doi: 10.1097/QAI.0b013e318256f3bc

Kessels, H. W., Nguyen, L. N., Nabavi, S., and Malinow, R. (2010). The prion protein as a receptor for amyloid-beta. *Nature* 466, E3–E4. doi: 10.1038/nature09217

Kudo, W., Petersen, R. B., and Lee, H. G. (2013). Cellular prion protein and Alzheimer disease: link to oligomeric amyloid-β and neuronal cell death. *Prion* 7, 114–116. doi: 10.4161/pri.22848

Kullmann, J. S., Grigoleit, J. S., Wolf, O. T., Engler, H., Oberbeck, R., Elsenbruch, S., et al. (2013). Experimental human endotoxemia enhances brain activity during social cognition. *Soc. Cogn. Affect. Neurosci*. doi: 10.1093/scan/nst049. [Epub ahead of print].

Kuwahara, C., Takeuchi, A. M., Nishimura, T., Haraguchi, K., Kubosaki, A., Matsumoto, Y., et al. (1999). Prions prevent neuronal cell-line death. *Nature* 400, 225–226. doi: 10.1038/22241.

Larson, M., Sherman, M. A., Amar, F., Nuvolone, M., Schneider, J. A., Bennett, D. A., et al. (2012). The complex PrP(c)-Fyn couples human oligomeric Aβ with pathological tau changes in Alzheimer's disease. *J. Neurosci.* 32, 16857–16871. doi: 10.1523/JNEUROSCI.1858-12.2012

Laurén, J., Gimbel, D. A., Nygaared, H. B., Gilbert, J. W., and Strittmatter, S. M. (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-β oligomers. *Nature* 457, 1128–1132. doi: 10.1038/nature07761

Lee, K. S., Chung, J. H., Lee, K. H., Shin, M. J., Oh, B. H., and Hong, C. H. (2008). Bioplex analysis of plasma cytokines in Alzheimer's disease and mild cognitive impairment. *Immunol. Lett.* 121, 105–109. doi: 10.1016/j.imlet.2008.09.004

Levesque, S., Surace, M. J., McDonald, J., and Block, M. L. (2011). Air pollution and the brain: subchronic diesel exhaust exposure causes neuroinflammation and elevates early markers of neurodegenerative disease. *J. Neuroinflammation* 8:105. doi: 10.1186/1742-2094-8-105

Levesque, S., Taetzsch, T., Lull, M. E., Johnson, J. A., McGraw, C., and Block, M. L. (2013). The role of MAC1 in diesel exhaust particle-induced microglial activation and loss of dopaminergic neuron function. *J. Neurochem.* 125, 756–765. doi: 10.1111/jnc.12231

Ley, E. J., Clond, M. A., Singer, M. B., and Salim, A. (2011). IL6 deficiency affects function after traumatic brain injury. *J. Surg. Res.* 170, 253–256. doi: 10.1016/j.jss.2011.03.006

Lucchini, R. G., Dorman, D. C., Elder, A., and Veronesi, B. (2012). Neurological impacts from inhalation of pollutants and the nose-brain connection. *Neurotoxicology* 33, 838–841. doi: 10.1016/j.neuro.2011.12.001

Malekzadeh, A., de Groot, V., Beckerman, H., van Oosten, B. W., Blankenstein, M. A., and Teunissen, C. (2012). Challenges in multiplex and monoplex platforms for the discovery of inflammatory profiles in neurodegenerative diseases. *Methods* 56, 508–513. doi: 10.1016/j.ymeth.2012.03.017

Martinez, A. N., Mehra, S., and Kaushal, D. (2013). Role of Interleukin-6 (IL6) in innate immunity to Mycobacterium tuberculosis infection. *J. Infect. Dis.* 207, 1253–1261. doi: 10.1093/infdis/jit037

Martins, V. R., Beraldo, F. H., Hajj, G. N., Lopes, M. H., Lee, K. S., Prado, M. A., et al. (2010). Prion protein: orchestrating neurotrophic activities. *Curr. Issues Mol. Biol.* 12, 63–86.

Massimino, M. L., Redaelli, M., Bertoli, A., Sorgato, M. C., and Mucignat-Caretta, C. (2013). Altered behavioral aspects of aged mice lacking the cellular prion protein. *Physiol. Behav.* 119C, 86–91. doi: 10.1016/j.physbeh.2013.06.006

Milhavet, O., and Lehmann, S. (2002). Oxidative stress and the prion protein in transmissible spongiform encephalopathies. *Brain Res. Brain Res. Rev.* 38, 328–339. doi: 10.1016/S0165-0173(01)00150-3

Mitsios, N., Saka, M., Krupinski, J., Pennucci, R., Sanfeliu, C., Turu, M. M., et al. (2007). Cellular prion protein is increased in the plasma and peri-infarcted brain tissue after acute stroke. *J. Neurosci. Res.* 85, 602–611. doi: 10.1002/jnr.21142

Molina, L. T., and Molina, M. J. (2004). Improving air quality in megacities: Mexico City case study. *Ann. N.Y. Acad. Sci.* 1023, 142–158. doi: 10.1196/annals.1319.006

Mooijaart, S. P., Sattar, N., Trompet, S., Lucke, J., Stott, D. J., Ford, I., et al. (2013). Circulating interleukin-6 concentration and cognitive decline in old age: the PROSPER study. *J. Intern. Med.* 274, 77–85. doi: 10.1111/joim.12052

Moon, H. Y., Kim, S. H., Yang, Y. R., Song, P., Yu, H. S., Park, H. G., et al. (2012). Macrophage migration inhibitory factor mediates the antidepressant actions of voluntary exercise. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13094–13099. doi: 10.1073/pnas.1205535109

Nunomura, A., Castellani, R. J., Lee, H. G., Moreira, P. I., Zhu, X., Perry, G., et al. (2006). Neuropathology in Alzheimer's disease: awakening from a hundred-year old dream. *Sci. Aging Knowledge Environ.* 2006, pe10. doi: 10.1126/sageke.2006.8.pe10

Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., et al. (2001). Oxidative damage is the earliest event in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.* 60, 759–767.

Nunomura, A., Tamaoki, T., Motohashi, N., Nakamura, M., McKeel, D. W. Jr., Tabaton, M., et al. (2012a). The earliest stage of cognitive impairment in transition from normal aging to Alzheimer's disease is marked by prominent RNA oxidation in vulnerable neurons. *J. Neuropathol. Exp. Neurol.* 71, 233–241. doi: 10.1097/NEN.0b013e318248e614

Nunomura, A., Moreira, P. I., Castellani, R. J., Lee, H. G., Zhu, X., Smith, M. A., et al. (2012b). Oxidative damage to RNA in aging and neurodegenerative disorders. *Neurotox. Res.* 22, 231–248. doi: 10.1007/s12640-012-9331-x

Nygaard, H. B., and Strittmatter, S. M. (2009). Cellular protein mediates the toxicity of beta-amyloid oligomers: implications for Alzheimer disease. *Arch. Neurol.* 66, 1325–1328. doi: 10.1001/archneurol.2009.223

Oh, J. M., Choi, E. K., Carp, R. I., and Kim, Y. S. (2012). Oxidative stress impairs autophagic flux in prion protein-deficient hippocampal cells. *Autophagy* 8, 1448–1461. doi: 10.4161/auto.21164

Ohta, S., Misawa, A., Fukaya, R., Inoue, S., Kanemura, Y., Okano, H., et al. (2012). Macrophage migration inhibitory factor (MIF) promotes cell survival and proliferation of neural stem/progenitor cells. *J. Cell Sci.* 125, 3210–3220. doi: 10.1242/jcs.102210

Papassotiropoulos, A., Hock, C., and Nitsch, R. M. (2001). Genetics of Interleukin 6: implications for Alzheimer's disease. *Neurobiol. Aging* 22, 863–871. doi: 10.1016/S0197-4580(01)00294-9

Peters, V. A., Joesting, J. J., and Freund, G. G. (2013). IL-1 receptor 2 (IL-1R2) and its role in immune regulation. *Brain Behav. Immun.* 32, 1–8. doi: 10.1016/j.bbi.2012.11.006

Popko, B. (2010). Myelin maintenance: axonal support required. *Nat. Neurosci.* 13, 275–277. doi: 10.1038/nn0310-275

Popp, J., Bacher, M., Kölsch, H., Noelker, C., Deuster, O., Dodel, R., et al. (2009). Macrophage inhibitory factor in mild cognitive impairment and Alzheimer's disease. *J. Psychiatr. Res.* 43, 749–753. doi: 10.1016/j.jpsychires.2008.10.006

Pradillo, J. M., Denes, A., Greenhalgh, A. D., Boutin, H., Drake, C., McColl, B. W., et al. (2012). Delayed administration of interleukin-1 receptor antagonist reduces ischemic brain damage and inflammation in comorbid rats. *J. Cereb. Blood Flow Metab.* 32, 1810–1819. doi: 10.1038/jcbfm.2012.101

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., et al. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55, 453–462. doi: 10.1002/glia.20467

Ramljak, S., Asif, A. R., Armstrong, V. W., Wrede, A., Groschup, M. H., Buschmann, A., et al. (2008). Physiological role of the cellular prion protein (PrPc): protein profiling study in two cell culture systems. *J. Proteome Res.* 7, 2681–2695. doi: 10.1021/pr7007187

Rezaie, P., Pontikis, C. C., Hudson, L., Caims, N. J., and Lantos, P. L. (2005). Expression of cellular prion protein in the frontal and occipital lobe in Alzheimer's disease, diffuse Lewy body disease, and in normal brain: an immunohistochemical

study. *J. Histochem. Cytochem.* 53, 929–940. doi: 10.1369/jhc.4A6551.2005

Rivest, S. (2009). Regulation of innate immune responses in the brain. *Nat. Rev. Immunol.* 9, 429–439. doi: 10.1038/nri2565

Rubio-Perez, J. M., and Morillas-Ruiz, J. M. (2012). A review: inflammatory process in Alzheimer's disease, role of cytokines. *ScientificWorldJournal* 2012:756357. doi: 10.1100/2012/756357

Saijo, E., Scheff, S. W., and Telling, G. C. (2011). Unaltered prion protein expression in Alzheimer disease patients. *Prion* 5, 109–116. doi: 10.4161/pri.5.2.16355

Savaskan, N. E., Fingerle-Rowson, G., Buchfelder, M., and Eyüpoglu, I. Y. (2012). Brain miffed by macrophage inhibitory factor. *Int. J. Cell Biol.* 2012:139573. doi: 10.1155/2012/139573

Scalabrino, G., and Veber, D. (2012). Normal prions as a new target of cobalamin (vitamin B12) in rat central nervous system. *Clin. Chem. Lab. Med.* 51, 601–606. doi: 10.1515/cclm-2012-0474

Schmidt, C., Artjomova, S., Hoeschel, M., and Zerr, I. (2013). CSF prion protein concentration and cognition in patients with Alzheimer disease. *Prion* 7, 229–234. doi: 10.4161/pri.23904

Schwarze-Eicker, K., Keyvani, K., Görtz, N., Westaway, D., Sachser, N., and Paulus, W. (2005). Prion protein (PrPc) promotes beta-amyloid plaque formation. *Neurobiol. Aging* 26, 1177–1182. doi: 10.1016/j.neurobiolaging.2004.10.004

Shameli, A., Yamanouchi, J., Tsai, S., Yang, Y., Clemente-Casares, X., Moore, A., et al. (2013). IL-2 promotes the function of memory-like autoregulatory CD8+T cells but suppresses their development via FoxP3+ Treg cells. *Eur. J. Immunol.* 43, 394–403. doi: 10.1002/eji.201242845

Smolen, J. S., Schoels, M. M., Nishmoto, N., Breedveld, F. C., Burmester, G. R., Dougados, M., et al. (2013). Consensus statement on blocking the effects of interleukin-6 and in particular by interleukin-6 receptor inhibition in rheumatoid arthritis and other inflammatory conditions. *Ann. Rheum. Dis.* 72, 482–492. doi: 10.1136/annrheumdis-2012-202469

Spittau, B., Zhou, X., Ming, M., and Krieglstein, K. (2012). IL6 protects MN9D cells and midbrain dopaminergic neurons from MPP+-induced neurodegeneration. *Neuromolecular Med.* 14, 317–327. doi: 10.1007/s12017-012-8189-7

Stellato, F., Spevacek, A., Proux, O., Minicozzi, V., Millhauser, G., and Morante, S. (2011). Zinc modulates copper coordination mode in prion protein octa-repeats subdomains. *Eur. Biophys. J.* 40, 1259–1270. doi: 10.1007/s00249-011-0713-4

Tokami, H., Ago, T., Sugimori, H., Kuroda, J., Awano, H., Suzuki, K., et al. (2013). RANTES has a potential to play a neuroprotective role in an autocrine/paracrine manner after ischemic stroke. *Brain Res.* 1517, 122–132. doi: 10.1016/j.brainres.2013.04.022

Tsai, D. H., Amvai, N., Marques-Vidal, P., Wang, J. L., Riediker, M., Mooser, V., et al. (2012). Effects of particulate matter on inflammatory markers in the general adult population. *Part. Fibre Toxicol.* 9, 24. doi: 10.1186/1743-8977-9-24

Tsutsui, S., Hahn, J. N., Johnson, T. A., Ali, Z., and Jirik, F. R. (2008). Absence of the cellular prion protein exacerbates and prolongs neuroinflammation in experimental autoimmune encephalomyelitis. *Am. J. Pathol.* 173, 1029–1041. doi: 10.2353/ajpath.2008.071062

Turtzo, L. C., Li, J., Persky, R., Benashski, S., Weston, G., Bucala, R., et al. (2013). Deletion of macrophage migration inhibitory factor worsens stroke outcome in female mice. *Neurobiol. Dis.* 54, 421–431. doi: 10.1016/j.nbd.2013.01.016

Um, J. W., Nygaard, H. B., Heiss, J. K., Kostylev, M. A., Stagi, M., Vortmeyer, A., et al. (2012). Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat. Neurosci.* 15, 1227–1235. doi: 10.1038/nn.3178

Uski, O. J., Happo, M. S., Jalava, P. I., Brunner, T., Kelz, J., Obemberger, I., et al. (2012). Acute systemic and lung inflammation in C57BI/6J mice after intratracheal aspiration of particulate matter from small-scale biomass combustion appliances based on old and modern technologies. *Inhal. Toxicol.* 24, 952–965. doi: 10.3109/08958378.2012.742172

Velayos, J. L., Irujo, A., Cuadrado-Tejedor, M., Paternain, B., Moleres, F. J., and Ferrer, V. (2009). The cellular prion protein and its role in Alzheimer disease. *Prion* 3, 110–117. doi: 10.4161/pri.3.2.9135

Viles, J. H., Cohen, F. E., Prusiner, S. B., Goodin, D. B., Wright, P. E., and Dyson, H. J. (1999). Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2042–2047. doi: 10.1073/pnas.96.5.2042

Voigtländer, T., Klöppel, S., Birner, P., Jarius, C., Flicker, H., Verghese-Nikolakaki, S., et al. (2001). Marked increase of neuronal prion protein immunoreactivity in Alzheimer's disease and human prion diseases. *Acta Neuropathol.* 101, 417–423.

Wang, J., Wicker, L. S., and Santamaria, P. (2009). IL-2 and its high-affinity receptor: genetic control of immunoregulation and autoimmunity. *Semin. Immunol.* 21, 363–371. doi: 10.1016/j.smim.2009.04.004

Watt, N. T., Griffiths, H. H., and Hooper, N. M. (2013). Neuronal zinc regulation and the prion protein. *Prion* 7, 203–208. doi: 10.4161/pri.24503

Westergard, L., Christensen, H. M., and Harris, D. A. (2007). The cellular prion protein (PrP^C): its physiological function and role in disease. *Biochim. Biophys. Acta* 1772, 629–644. doi: 10.1016/j.bbadis.2007.02.011

Whitehouse, I. J., Jackson, C., Turner, A. J., and Hooper, N. M. (2010). Prion protein is reduced in aging and in speoradic but not in familial Alzheimer's disease. *J. Alzheimers Dis.* 22, 1023–1031.

Whitehouse, I. J., Miners, J. S., Glennon, E. B., Kehoe, P. G., Love, S., Kellet, K. A., et al. (2013). Prion protein is decreased in Alzheimer's brain and inversely correlates with BACE1 activity, amyloid-β levels and Braak stage. *PLoS ONE* 8:e59554 doi: 10.1371/journal.pone.0059554

Willette, A. A., Bendlin, B. B., McLaren, D. G., Canu, E., Kastman, E. K., Kosmatka, K. J., et al. (2010). Age-related changes in neural volume and microstructure associated with interleukin-6 are ameliorated by a calorie-restricted diet in old rhesus monkeys. *Neuroimage* 51, 987–994. doi: 10.1016/j.neuroimage.2010.03.015

Wittkopp, S., Staimer, N., Tjoa, T., Gillen, D., Daher, N., Shafer, M., et al. (2013). Mitochondrial genetic background modifies the relationship between traffic-related air pollution exposure and systemic biomarkers of inflammation. *PLoS ONE* 8:e64444. doi: 10.1371/journal.pone.0064444

Yan, J., Liu, J., Lin, C. Y., Anzgene, A. N., Csurhes, P. A., Pender, M. P., et al. (2012). Interleukin-6 gene promoter-572 C allele may play a role in rate of disease progression in multiple sclerosis. *Int. J. Mol. Sci.* 13, 13667–13679. doi: 10.3390/ijms131013667

Younan, N. D., Sarell, C. J., Davies, P., Brown, D. R., and Viles, J. H. (2013). The cellular prion protein traps Alzheimer's Aβ in an oligomeric form and disassembles amyloid fibers. *FASEB J.* 27, 1847–1858. doi: 10.1096/fj.12-222588

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 July 2013; accepted: 23 September 2013; published online: 10 October 2013.

Citation: Calderón-Garcidueñas L, Cross JV, Franco-Lira M, Aragón-Flores M, Kavanaugh M, Torres-Jardón R, Chao C-k, Thompson C, Chang J, Zhu H and D'Angiulli A (2013) Brain immune interactions and air pollution: macrophage inhibitory factor (MIF), prion cellular protein (PrP^C), Interleukin-6 (IL-6), interleukin 1 receptor antagonist (IL-1Ra), and interleukin-2 (IL-2) in cerebrospinal fluid and MIF in serum differentiate urban children exposed to severe vs. low air pollution. Front. Neurosci. 7:183. doi: 10.3389/fnins.2013.00183

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.

frontiers in
NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 17 September 2013
doi: 10.3389/fnins.2013.00158

Inflammatory macrophage phenotype in BTBR T+tf/J mice

Charity E. Onore[1,2], Milo Careaga[1,2], Brooke A. Babineau[3,4], Jared J. Schwartzer[1,5], Robert F. Berman[1,5] and Paul Ashwood[1,2]*

[1] *The MIND Institute, University of California, Davis, CA, USA*
[2] *Department of Medical Microbiology and Immunology, University of California, Davis, CA, USA*
[3] *Department of Pediatrics, University of California, San Francisco, CA, USA*
[4] *Institute for Human Genetics, University of California, San Francisco, CA, USA*
[5] *Department of Neurological Surgery, University of California, Davis, CA, USA*

Edited by:
Adam Denes, University of Manchester, UK

Reviewed by:
Jaleel A. Miyan, The University of Manchester, UK
Sylvie Girard, University of Manchester, UK

****Correspondence:***
Paul Ashwood, The MIND Institute, University of California, 2825 50th Street, Sacramento, CA 95817, USA
e-mail: pashwood@ucdavis.edu

Although autism is a behaviorally defined disorder, many studies report an association with increased pro-inflammatory cytokine production. Recent characterization of the BTBR T+tf/J (BTBR) inbred mouse strain has revealed several behavioral characteristics including social deficits, repetitive behavior, and atypical vocalizations which may be relevant to autism. We therefore hypothesized that, asocial BTBR mice, which exhibit autism-like behaviors, may have an inflammatory immune profile similar to that observed in children with autism. The objectives of this study were to characterize the myeloid immune profile of BTBR mice and to explore their associations with autism-relevant behaviors. C57BL/6J (C57) mice and BTBR mice were tested for social interest and repetitive self-grooming behavior. Cytokine production was measured in bone-marrow derived macrophages incubated for 24 h in either growth media alone, LPS, IL-4/LPS, or IFNγ/LPS to ascertain any M1/M2 skewing. After LPS stimulation, BTBR macrophages produced higher levels of IL-6, MCP-1, and MIP-1α and lower IL-10 ($p < 0.01$) than C57 mice, suggesting an exaggerated inflammatory profile. After exposure to IL-4/LPS BTBR macrophages produced less IL-10 ($p < 0.01$) than C57 macrophages and more IL-12p40 ($p < 0.01$) suggesting poor M2 polarization. Levels of IL-12(p70) ($p < 0.05$) were higher in BTBR macrophages after IFNγ/LPS stimulation, suggesting enhanced M1 polarization. We further observed a positive correlation between grooming frequency, and production of IL-12(p40), IL-12p70, IL-6, and TNFα ($p < 0.05$) after treatment with IFNγ/LPS across both strains. Collectively, these data suggest that the asocial BTBR mouse strain exhibits a more inflammatory, or M1, macrophage profile in comparison to the social C57 strain. We have further demonstrated a relationship between this relative increase in inflammation and repetitive grooming behavior, which may have relevance to repetitive and stereotyped behavior of autism.

Keywords: autism, BTBR, behavior, immune system, inflammation, macrophage, M1, M2

BACKGROUND

Autism is a behaviorally defined disorder without a known physiological cause. This disorder is characterized by social deficits, communication deficits, and the presence of repetitive or stereotyped behaviors (APA, 2000). Several studies have attempted to identify a genetic cause for autism (Yrigollen et al., 2008; Voineagu et al., 2011; Lintas et al., 2012); however, the majority of autism cases remain idiopathic in nature (Abrahams and Geschwind, 2008). Although the genetic cause for autism remains undetermined, there is increasing evidence that immune function may play a role in the disorder. A number of studies have identified trends in abnormal immune function in individuals with autism including a trend toward high cytokine production and atypical immune cell function (Ashwood et al., 2006, 2011a,b,c; Enstrom et al., 2010). There is further evidence suggesting that many of these atypical immune profiles are associated with worsening autism-associated behaviors (Onore et al., 2012).

Immune abnormalities in autism spectrum disorders have been characterized in a number of studies, including evidence of increased microglia and astroglia activation in the brain, as well as increased levels of interferon (IFN)γ, interleukin (IL)-1β, IL-6, IL-12, tumor necrosis factor (TNF)-α, and macrophage chemotactic protein (MCP)-1 in brain tissue and cerebral spinal fluid (Vargas et al., 2005; Li et al., 2009; Morgan et al., 2010). In parallel with findings within the central nervous system (CNS), increased plasma levels of these cytokines including IL-1β, IL-6, IL-12p40, and MCP-1 have been reported as well (Ashwood et al., 2011b). Of note, peripheral plasma levels of these cytokines were associated with worsening autism-associated behaviors in the areas of communication and social interaction, suggesting a relationship between peripheral immune activity and behavioral symptoms of the disorder (Ashwood et al., 2011b,c).

Given the unknown pathophysiology of autism and highly variable genetics of the disorder, an animal model that exhibits

behavioral characteristics with relevance to autism-features rather than a direct genetic link may be useful for examining potential relationship between physiology and behavior. The BTBR T+tf/J mouse (referred to as BTBR here-in) is an inbred strain that has recently been described to exhibit behaviors relevant to autism, including asocial behavior, repetitive behavior, and atypical vocalizations (Moy et al., 2007; McFarlane et al., 2008; Scattoni et al., 2008, 2011). There is also evidence to suggest that the BTBR mouse exhibits increased ERK signaling (Zou et al., 2011), a common finding in individuals with Fragile X Syndrome, one of the leading known genetic risk factors for autism. However, little is currently known about the relationship between BTBR associated behavior and immunity in the BTBR mouse. Recent research has outlined differences in immune function including increased titers of immunoglobulin (Ig) and inflammatory cytokine levels in brain tissue, and greater susceptibility to listeriosis than C57Bl/6J mice (referred to as C57 here-in) (Heo et al., 2011). Immunity against listeriosis is largely based on the ability of macrophages to polarize to an inflammatory (M1) profile (Pfeffer et al., 1993; Jouanguy et al., 1999; Benoit et al., 2008). This data suggests immunological differences between the two strains and may implicate aberrant macrophage function, but there is as yet no direct evidence of a relationship between the function of the immune cells and behavioral symptoms in the BTBR mouse.

We hypothesized that there are associations between myeloid inflammation and autism relevant behaviors in the BTBR mouse. To further elucidate the relationship between inflammation and behavior, and to examine the role of M1/M2 polarization in BTBR, we analyzed individual mice for social interaction and repetitive grooming behavior and measured macrophage cytokine production *in vitro*. Bone marrow-derived macrophages were generated for each mouse and tested for inflammatory and anti-inflammatory cytokine responses. In addition, we further characterized immune function by testing the ability of BTBR macrophages to polarize to pro-inflammatory IL-12 high (M1) and anti-inflammatory IL-10 high (M2) macrophage subtypes *in vitro* (Mantovani et al., 2005).

METHODS

MICE

C57Bl/6J (C57) mice (Jackson Laboratory-West, Sacramento, CA), BTBR T^{+}tf/J (BTBR) mice (Jackson Laboratory, Bar Harbor, ME), and 129/SvImJ (129) mice (Jackson Laboratory) were maintained by the Campus Laboratory Animal Services, at University of California, Davis at ambient room temperature on a 12 h light/dark cycle. Food and water were provided *ad libitum*. All procedures were performed with approval by the University of California, Davis Institutional Animal Care and Use Committee and in accordance with the guidelines provided by the National Institutes of Health for the humane treatment of animals. Social approach and self-grooming assays were conducted in dedicated behavioral testing rooms during the standard light phase, usually between 1000 and 1500 h. Mice of the 129 strain were used as novel mouse controls in the social approach testing paradigm. Mice were deeply anesthetized with isofluorane and euthanized by decapitation. Femurs and tibia were aseptically removed and stored in RPMI 1640 (Life Technologies) media supplemented with 10% FBS, 100 IU/ml penicillin, 100 IU/ml streptomycin, 25 μg/ml gentimycin (Sigma) prior to processing. For this study, nine C57 mice (male, age 10–12 weeks) and seven BTBR mice (male, age 10–12 weeks), were utilized.

SOCIAL APPROACH

Social approach was assayed in an automated three-chambered apparatus (NIMH Research Services Branch, Bethesda, MD) using methods previously described (Crawley, 2007; Yang et al., 2007, 2009, 2011a,b; Chadman et al., 2008; McFarlane et al., 2008; Moy et al., 2008; Silverman et al., 2010, 2011). Briefly, the apparatus was a rectangular, three-chambered box made of clear polycarbonate. Photocells embedded in the doorways automatically detected entries between chambers and the amount of time spent in each chamber by the subject mouse. The test session began with a 10 min habituation session in the center chamber only, followed by a 10 min habituation to all three empty chambers. At the completion of the second habituation phase, the subject mouse was returned to the center chamber and a clean novel object (wire cup) was placed in one of the side chambers and a novel 129 mouse of similar age and weight was placed in an identical wire cup located in the other side chamber. After both stimuli were positioned, the subject mouse was allowed access to all three chambers for 10 min. Trials were video recorded and time spent sniffing the novel object and time spent sniffing the novel mouse were later scored by a trained observer using a handheld stopwatch. Performance on social approach was plotted as a "social score," which is equal to the amount of time (in seconds) the experimental animal spent sniffing the novel mouse minus the time spent sniffing the novel object. Therefore, high positive scores indicate high sociability, and low or negative scores indicate low sociability.

SPONTANEOUS SELF GROOMING

Mice were scored for spontaneous self-grooming behaviors as described previously (Yang et al., 2007, 2009; McFarlane et al., 2008; Silverman et al., 2010). Briefly, each mouse was given a 10 min habituation in a clean, empty mouse cage and then video recorded for 10 min. The video recorded session was scored for cumulative time spent grooming all body regions by a trained observer using a stopwatch. Differences in color and markings between the inbred strains prevented fully blind ratings. However, the distinguishing features of the strain were less visible in the video recordings, which is why this method was chosen over live scoring. The animals' behavior in the spontaneous self-grooming tasks was plotted as "grooming" which indicates the amount of time (in seconds) the experimental animal spent grooming itself during the 10-min observation phase.

GENERATION OF MACROPHAGE MEDIA

Confluent L929 Cells (ATCC, Manassas, VA) were cultured for 7 days in complete Dubelcco's modified Eagles media (DMEM) F-12 (Life Technologies, Carlsbad, CA) supplemented with 10% low endotoxin, heat inactivated fetal bovine serum (FBS) (Life technologies), 100 IU/ml penicillin, and 100 IU/ml streptomycin (Sigma, St Louis, MO). The resulting L929 conditioned media was passed through a 2 μm filter (Millipore, Billerica, MA) to remove

cellular debris and ensure sterility. To create macrophage media, complete DMEM was supplemented 10% with filtered L929 conditioned media. L929 conditioned media was stored at −20°C for less than 60 days before single thaw and use.

BONE MARROW-DERIVED MACROPHAGE GENERATION

Legs with fur and skin removed, including femur and tibia from each mouse, were washed twice in sterile cold (4°C) Hanks buffered saline solution (HBSS) (Mediatech, Herndon, VA). Tissue was removed from the bones with sterile scissors and forceps, and bones were washed in 10 mls cold HBSS. The proximal and distal ends of both the femur and tibia were removed, and the lumens of the femurs and tibia were flushed with 10 mls of cold HBSS using a 25 gauge needle (BD Medical, Franklin Lakes, NJ). Dislodged bone marrow was agitated by aspiration and ejected with a 22 gauge needle (BD Medical). The resulting cell suspension was filtered through a 100 μm nylon mesh (BD Biosciences, Carlsbad, CA). Cells were pelleted by centrifugation at 500 g for 5 min and resuspended in macrophage media to a concentration of 1×10^5 cells/ml, plated in sterile non-cell culture treated petri dishes (BD Biosciences) at a volume of 10 mls per dish and incubated for 3 days at 37°C, 5% CO_2. Five mls of macrophage media was then added to each dish and cells were incubated for a further 4 days at 37°C, 5% CO_2 for a total incubation time of 7 days. Petri dishes containing adherent, mature bone marrow-derived macrophages were washed with 10 mls cold HBSS, and incubated with 3 mls Cell Stripper™ buffer (Mediatech) per plate, for 5 min at 37°C, 5% CO_2. Following incubation, cells were dislodged from the petri dishes by gentle pipetting, diluted in an equal amount of complete DMEM, and pelleted by centrifugation at 400 g for 5 min. Cells were resuspended at 1×10^6 cell/ml in complete DMEM, and 1 ml was plated in 12-well sterile tissue culture plate (Greiner Bio-One, Monroe, NC) and allowed to adhere overnight at 37°C, 5% CO_2. Following adhesion of bone marrow-derived macrophages, complete DMEM was aspirated and replaced with 1 ml/well of 1×10^6 cells with the following eight conditions: media alone, 2 ng/ml recombinant mouse IL-4 (R&D, Minneapolis, MN), 150 ng/ml recombinant mouse IFNγ (R&D), 10 ng/ml lipopolysaccharide (LPS) (Sigma), 2 ng/ml recombinant mouse IL-4 with 10 ng/ml LPS, or 150 ng/ml recombinant mouse IFNγ with 10 ng/ml LPS. Cells were incubated under these conditions for 24 h, at which point supernatants were collected and stored at −80°C until assayed.

CYTOKINE MEASUREMENT

The quantification of the cytokines Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), IFNγ, IL-1β, IL-6, IL-10, IL-13, IFNγ induced protein (IP)-10 (CXCL10), MCP-1 (CCL2), TNFα, macrophage inflammatory protein (MIP)-1α (CCL3), and MIP-1β (CCL4) in supernatants was determined using mouse specific Milliplex™ multiplexing bead immunoassays (Millipore, Billerica, MA). The cytokines IL-12p40 and IL-12p70 were measured using Bioplex™ multiplexing bead immunoassays (Bio-Rad Laboratories, Hercules, CA). Samples were run in accordance with the instructions of the manufactures protocol. In brief, 25 μL of supernatant was incubated with antibody-coupled beads. After a series of washes, a biotinylated detection antibody was added to the beads, and the reaction mixture was detected by the addition of streptavidin conjugated to phycoerythrin. The bead sets were analyzed using a flow-based Luminex™ 100 suspension array system (Bio-Plex 200; Bio-Rad Laboratories). Unknown sample cytokine concentrations were calculated by Bio-Plex Manager software using a standard curve derived from the known reference cytokine concentrations supplied by the manufacturer. A five-parameter model was used to calculate final concentrations and values are expressed in pg/ml. The sensitivity of this multiplex immunoassay allowed the detection of cytokine concentrations with the following minimal detectable limits: IL-1β (2 pg/ml), IL-6 (1.8 pg/ml), IL-10 (3.3 pg/ml), IL-12(p40) (1.53 pg/ml), IL-12(p70) (1.62 pg/ml), IFNγ (0.9 pg/ml), IP-10 (0.6 pg/ml), MCP-1 (5.3 pg/ml), MIP-1α (8.7 pg/ml), MIP-1β (10.1 pg/ml), and TNFα (1 pg/ml).

STATISTICAL ANALYSIS

Statistical analysis to compare cytokine levels between BTBR and C57 groups was conducted with Mann–Whitney test. Correlation analysis was performed with Spearman correlation analysis. All analyses were two-tailed, and values of $p < 0.05$ were considered statistically significant. Unadjusted p-values are presented for multiplex cytokine data (Rothman, 1990). Medians and interquartile ranges (IQR) are reported for all measured cytokines and chemokines. All analyses were conducted with GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA).

RESULTS

CYTOKINE RESPONSES

To determine the cytokine response to LPS (a gram negative bacterial endotoxin) with and without polarization, bone marrow macrophages were incubated in media alone, IL-4 (a M2 polarizing cytokine), or IFNγ (a M1 polarizing cytokine) with LPS. Even without presence of polarizing cytokine or LPS stimulus, BTBR macrophages exhibit a trend toward higher inflammatory cytokine production, with significantly higher production of IL-12(p40) [C57 median ± IQR: 15.30 ± 7.56; median ± IQR: 20.58 ± 7.56; $p = 0.0485$] (**Table 1**). Following stimulation with LPS, BTBR macrophages produce significantly higher levels of inflammatory cytokines IL-6 [C57 median ± IQR: 1254 ± 760.2; BTBR median ± IQR: 3112 ± 1189; $p = 0.0003$], IL-12(p40) [C57 median ± IQR: 446.4 ± 322.5; BTBR median ± IQR: 2779 ± 1652; $p = 0.0002$], MCP-1 [C57 median ± IQR: 750.4 ± 442.6; BTBR median ± IQR: 3117 ± 1036; $p = 0.0002$], IP-10 [C57 median ± IQR: 8875 ± 2859; BTBR median ± IQR: 14535 ± 1294; $p = 0.0007$], and MIP-1α [C57 median ± IQR: 1945 ± 713; BTBR median ± IQR: 4342 ± 2828; $p = 0.0002$] and lower levels of anti-inflammatory cytokine IL-10 [C57 median ± IQR: 448.4 ± 307.6; BTBR median ± IQR: 284.2 ± 195.2; $p = 0.0229$] (**Table 1**).

To determine whether BTBR macrophages are more inclined toward a pro-inflammatory M1 phenotype, we measured the levels of M1 and M2 associated cytokines following polarization with IL-4 and LPS to induce a M2 phenotype or IFNγ and LPS to induce a M1 phenotype. Our initial experiments revealed treatment with IL-4 alone was not sufficient to induce a cytokine

Table 1 | Macrophage cytokine levels in unstimulated and LPS stimulated culture conditions.

Cytokines pg/ml	Unstimulated: Median (IQR)			LPS: Median (IQR)		
	C57	BTBR	*p*-value	C57	BTBR	*p*-value
IL-1β	3.9 (14.39)	BLD	N/A	BLD	16.56 (38.26)	0.351
IL-6	0.23 (2.22)	BLD	N/A	1254 (760.2)	3112 (1189)	0.0003*
IL-10	BLD	BLD	N/A	448.4 (307.6)	284.2 (195.2)	0.023*
IL-12p40	15.3 (7.56)	20.58 (7.56)	0.049*	446.4 (322.5)	2779 (1652)	0.0002*
IL-12p70	BLD	BLD	N/A	BLD	56.81 (56.81)	N/A
IP-10	65.45 (30.94)	70.17 (93.81)	0.142	8875 (2859)	14535 (1294)	0.0007*
MCP-1	BLD	15.63 (32.74)	N/A	750.4 (442.6)	3117 (1036)	0.0002*
TNF-α	BLD	0.13 (5.1)	N/A	448.3 (407.5)	595.2 (217.2)	0.408
MIP-1α	39.27 (22.41)	BLD	N/A	1945 (713)	4342 (2828)	0.0002*
MIP-1β	63.35 (36.58)	56.76 (83.06)	0.671	10214 (2530)	23060 (9262)	0.0003*

Median cytokine measurements and interquartile ranges (IQR) in pg/ml. All p-values were calculated by two-tailed Mann Whitney U-test.

**Significant p-values; BLD: Below level of detection; N/A: Not applicable, p-values could not be calculated.*

response distinguishable from that of media alone. Following stimulation with IL-4 and LPS, BTBR macrophages produce significantly lower levels of IL-10 [C57 median ± IQR: 641.9 ± 164.2; BTBR median ± IQR: 222.6 ± 83.4; $p = 0.0.0003$], a M2 cytokine, and higher IL-12p40 [C57 median ± IQR: 182.3 ± 102.6; BTBR median ± IQR: 613.8 ± 359.4; $p = 0.001$], IL-6 [C57 median ± IQR: 558.6 ± 288.1; BTBR median ± IQR: 1264 ± 330; $p = 0.0052$], and MIP-1α [C57 median ± IQR: 2551 ± 1150; BTBR median ± IQR: 4179 ± 4467; $p = 0.0021$]. Following stimulation with IFNγ and LPS, BTBR macrophages produce significantly higher levels of IL-12(p40) [C57 median ± IQR: 12496 ± 5494; BTBR median ± IQR: 18607 ± 326.6; $p = 0.0401$], IL-12(p70) [C57 median ± IQR: 251.9 ± 302.17; BTBR median ± IQR: 625.5 ± 477.1; $p = 0.0108$], and IP-10 [C57 median ± IQR: 3464 ± 1953; BTBR median ± IQR: 7497 ± 3553; $p = 0.0002$] but also more IL-10 [C57 median ± IQR: 35.24 ± 15.36; BTBR median ± IQR: 74.8 ± 30.9; $p = 0.0115$] and MCP-1 [C57 median ± IQR: 1238 ± 802; BTBR median ± IQR: 1954 ± 752; $p = 0.0012$] (**Table 2**). BTBR macrophages appeared to exhibit an overall trend toward increased M1 polarization (**Figure 1**).

BEHAVIORAL AND IMMUNE CORRELATIONS

To examine a potential relationship between the immune profile observed in BTBR macrophages, and the impaired social interaction and repetitive grooming associated with the BTBR strain, we tested for associations by Spearman analysis. There was very little association between cytokine profiles and social behavior in the BTBR mouse; however, for macrophage cell cultures, IL-1β demonstrated a negative association with sociability after IFNγ/LPS incubation [$r = -0.56$; $p = 0.023$] such that as IL-1β levels increased social approach decreased (**Figure 2**). In contrast, grooming behavior was associated with a large number of cytokines including associations with IL-12(p40) [$r = 0.58$; $p = 0.033$] and IP-10 [$r = 0.55$; $p = 0.032$] in unstimulated macrophages (**Figure 3**) suggesting that as cytokine levels increased there was an increase in repetitive behaviors. Following stimulation with LPS, inflammatory cytokines IL-6 [$r = 0.77$; $p = 0.001$] and MIP-1β [$r = 0.67$; $p = 0.006$] were positively associated with grooming behavior, while the anti-inflammatory M2 associated cytokine IL-10 demonstrated a negative association with grooming [$r = -0.63$; $p = 0.012$] (**Figure 4**). This data suggests that inflammatory cytokines are associated with more impaired repetitive behavior whereas anti-inflammatory cytokines are associated with improvement in behaviors. In polarization conditions such as IL-4/LPS incubation, IL-10 again was negatively associated with grooming [$r = -0.86$; $p = 0.0001$], while IL-6 [$r = -0.63$; $p = 0.012$], IP-10 [$r = 0.775$, $p = 0.0007$], and MIP-1β [$r = 0.5286$, $p = 0.0428$] were positively associated with grooming time (**Figure 5**). Macrophage cytokines produced following incubation with the M1 polarizing IFNγ/LPS condition including IL-12(p40) [$r = 0.72$; $p = 0.004$], IL-12(p70) [$r = 0.77$; $p = 0.001$], and TNF-α [$r = 0.63$; $p = 0.011$] also positively associated with increased grooming behavior following incubation with the M1 polarizing IFNγ/LPS condition, such that M1 macrophage cytokine production was associated with more impaired behavior. Interestingly, within the BTBR strain alone, IL-12(p70) [$r = 0.87$; $p = 0.03$] and TNF-α [$r = 0.94$; $p = 0.005$] levels were also positively correlated with grooming (**Figure 6**).

DISCUSSION

In this study we describe several immune features in the asocial BTBR mouse that differ from social C57 mice including higher production of inflammatory cytokines and chemokines in bone-marrow derived macrophages. BTBR macrophages produced increased levels of IL-6 after LPS stimulation. Even without stimulation, BTBR macrophages produced higher levels of IL-12(p40) and IP-10, suggesting a possible M1 polarization even without the presence of M1 polarizing exposure to IFNγ or LPS. After stimulation with LPS, IL-12(p40) remained higher in BTBR mice, while IL-10 was lower, again suggesting M1 polarization even in the absence of IFNγ. Levels of IL-12(p70) were undetectable in C57 mice, but were detectable in the majority of BTBR samples, suggesting IL-12(p70) levels may also be higher in BTBR mice. However, given that IL-12(p70) were below levels of detection in the C57, statistical analysis was limited and *p*-values could not be calculated. In addition to evidence of M1

Table 2 | Macrophage cytokine levels in IL-4/LPS and IFNγ/LPS stimulated culture conditions.

Cytokines pg/ml	IL-4/LPS: Median (IQR)			IFNγ/LPS: Median (IQR)		
	C57	BTBR	*p*-value	C57	BTBR	*p*-value
IL-1β	20.04 (32.39)	BLD	0.252	BLD	46.36 (28.18)	N/A
IL-6	558.6 (288.1)	1264 (330)	0.005*	10680 (8041)	10667 (5695)	0.919
IL-10	641.9 (164.2)	222.6 (83.4)	0.0003*	35.24 (15.36)	74.8 (30.9)	0.012*
IL-12p40	182.3 (102.6)	613.8 (359.4)	0.001*	12496 (5494)	18607(326.6)	0.040*
IL-12p70	BLD	BLD	N/A	251.9 (302.17)	625.5 (477.1)	0.011*
IP-10	3464 (1953)	7497 (3553)	0.0002*	18319 (7141)	16726 (10137)	0.351
MCP-1	2692 (682)	3373 (1065)	0.351	1238 (802)	1954 (752)	0.001*
TNF-α	418.2 (292.6)	498.5 (282)	0.351	975.2 (451.8)	1393 (974)	0.071
MIP-1α	2551 (1150)	4179 (4467)	0.002*	872.3 (321.6)	1134 (330.2)	0.091
MIP-1β	4264 (2097)	6136 (17675)	0.055	4019 (1492)	5791 (3024)	0.071

Median cytokine measurements and interquartile ranges (IQR) in pg/ml. All p-values were calculated by two-tailed Mann Whitney U-test.

**Significant p-values; BLD: Below level of detection; N/A: Not applicable, p-values could not be calculated.*

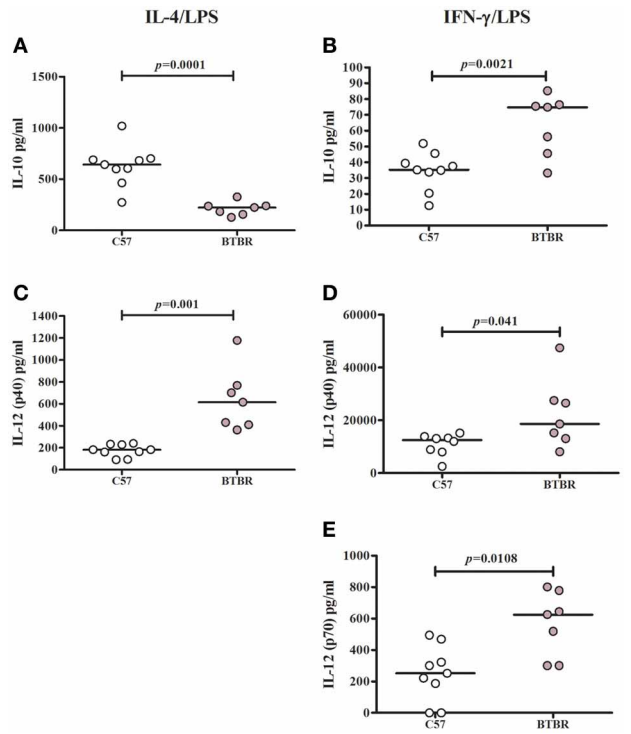


FIGURE 1 | M1 and M2 cytokine polarization in BTBR and C57 macrophages. Scatter graphs and median values of IL-10 **(A,B)**, IL-12p40 **(C,D)** and IL-12p70 **(E)** after IL-4/LPS incubation **(A,C)** and IFNγ/LPS incubation **(B,D,E)**. IL-12(p70) values were below observable levels in IL-4/LPS stimulated samples. *p*-values were calculated with Mann–Whitney test.

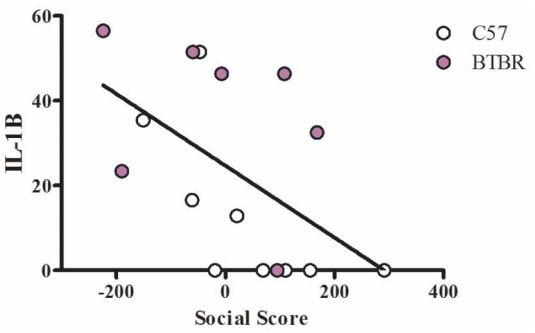


FIGURE 2 | Social correlation in IFNγ/LPS stimulated macrophages. Scatter graphs and linear regression of IL-1β in IFNγ stimulated macrophages. Correlation coefficients (*r*-values) and *p*-values were calculated with Spearman's correlation analysis. C57 *r*-value: −0.80, *p*-value: 0.009*; BTBR *r*-value: −0.43, *p*-value: 0.333; Across strains *r*-value: −0.56, *p*-value: 0.023*; *significant *p*-values.

polarization, levels of chemokines MIP-1α and MCP-1 were also higher in BTBR macrophages after LPS stimulation suggesting chemotactic responses may also be enhanced in this asocial mouse strain.

Macrophage polarization has many similarities to T helper (T_H) cell subtypes and can be divided into two major groups, denoted M1 and M2 (Mantovani et al., 2005; Mosser and Edwards, 2008). M1 macrophages are IL-12^{high}, IL-10^{low}, they are higher inflammatory cytokine producers than their M2 counterparts, and promote a T_H1 response in part through production of IL-12(p70). IL-12(p40) is a subunit of both IL-12(p70) and IL-23, which are both produced in significant quantities by M1 macrophages (Oppmann et al., 2000). While M2 cells can also produce inflammatory cytokines, they tend to produce far less than M1 macrophages, and have an IL-12^{low}, IL-10^{high} phenotype, and promote T_H2 responses. M1 polarization has been implicated in a number of neurological disease including multiple sclerosis and Alzheimer's disease (Mikita et al., 2011), and recent literature suggests that promoting an M2 phenotype may be beneficial to cognitive function (Derecki et al., 2010a). Transplant of wild-type IL-4 competent T cells in to IL-4$^{-/-}$ mice results in improved cognition (Derecki et al., 2010b), and transplant of M2 polarized macrophages results in improved cognitive performance in immune deficient mice (Derecki et al., 2011). Together, this data suggests that M1 macrophage polarization may have

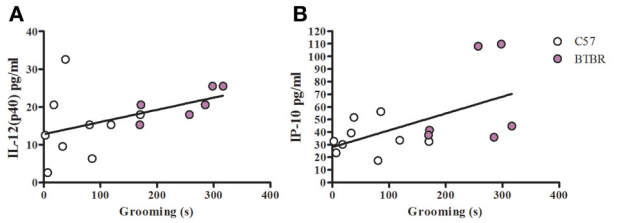


FIGURE 3 | Grooming correlation in unstimulated macrophages. Scatter graphs and linear regression of IL-12(p40) **(A)** and IP-10 **(B)** in unstimulated macrophages. Correlation coefficients (*r*-value) and *p*-values were calculated with Spearman's correlation analysis. **(A)** C57 *r*-value: 0.27, *p*-value: 0.493; BTBR *r*-value: 0.88, *p*-value: 0.033*; Across strains *r*-value; 0.58, *p*-value: 0.024* **(B)** C57 *r*-value: 0.23, *p*-value: 0.546; BTBR *r*-value: 0.43, *p*-value: 0.397; Across strains *r*-value: 0.55, *p*-value: 0.032*; *significant *p*-values.

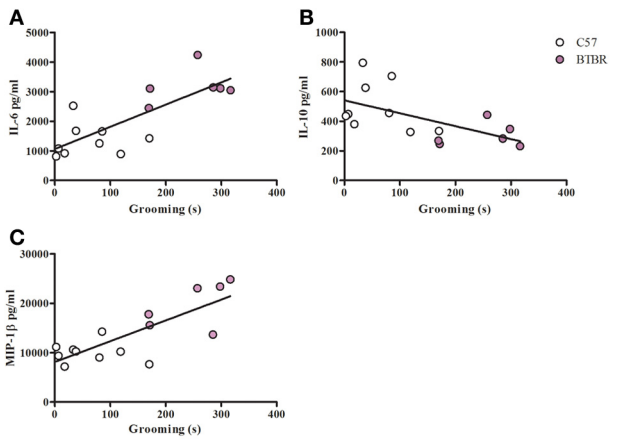


FIGURE 4 | Grooming correlation in LPS stimulated macrophages. Scatter graphs and linear regression of IL-6 **(A)**, IL-10 **(B)**, and **(C)** MIP-1β in LPS stimulated macrophages. Correlation coefficients (*r*-values) and *p*-values were calculated with Spearman's correlation analysis. **(A)** C57 *r*-value: 0.30, *p*-value: 0.433; BTBR *r*-value: 0.20, *p*-value: 0.704; Across strains *r*-value: 0.77, *p*-value: 0.001* **(B)** C57 *r*-value: −0.22, *p*-value: 0.576; BTBR *r*-value: −0.09, *p*-value: 0.872; Across strains *r*-value: −0.63, *p*-value: 0.012* **(C)** C57 *r*-value: −0.15, *p*-value: 0.708; BTBR *r*-value: 0.60, *p*-value: 0.241; Across strains *r*- value: 0.67, *p*-value: 0.006; *significant *p*-values.

detrimental effects on normal brain function whilst M2 can ameliorate some of these effects.

To further examine the proclivity of BTBR macrophages to polarize to an M1 phenotype, C57 and BTBR macrophages were stimulated with LPS in the presence of IFNγ (M1) or IL-4 (M2) (Zhang et al., 2008). Under M1 polarizing conditions, BTBR macrophages produced significantly higher IL-12(p40) and IL-12(p70), mirroring the enhanced M1 polarization. In addition under M2 polarizing conditions, BTBR macrophages produced significantly less IL-10, and significantly more IL-12(p40) suggesting poor relative M2 polarization compared to C57 mice (**Figure 1**). Collectively, these data suggest that BTBR macrophages are innately M1 skewed, and more inclined to produce M1 associated cytokines such as IL-12(p70).

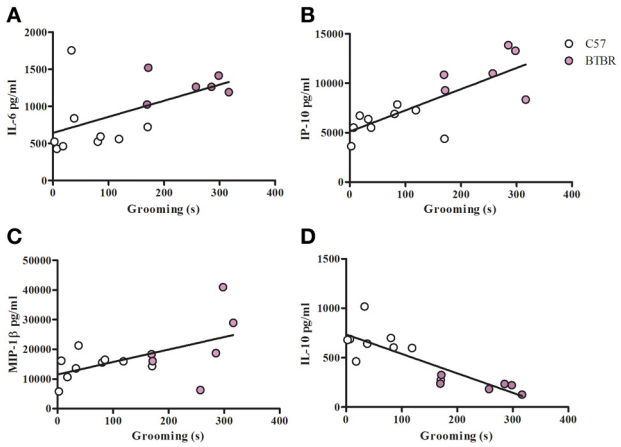


FIGURE 5 | Grooming correlation in IL-4/LPS stimulated macrophages. Scatter graphs and linear regression of IL-6 **(A)**, IP-10 **(B)**, MIP-1β **(C)**, and IL-10 **(D)** in IL-4/LPS stimulated macrophages. Correlation coefficients (*r*-value) and *p*-values were calculated with Spearman's correlation analysis. **(A)** C57 *r*-value: 0.43, *p*-value: 0.244; BTBR *r*-value: 0.09, *p*-value: 0.872; Across strains *r*-value: 0.63, *p*-value: 0.012* **(B)** C57 *r*-value: 0.40, *p*-value: 0.291; BTBR *r*-value: 0.02, *p*-value: 1; Across strains *r*-value: 0.78, *p*-value: 0.0007* **(C)** C57 *r*-value: 0.38, *p*-value: 0.312; BTBR *r*-value: 0.71, *p*-value: 0.136; Across strains *r*-value: 0.53, *p*-value: 0.042* **(D)** C57 *r*-value: −0.48, *p*-value: 0.188; BTBR *r*-value: −0.77, *p*-value: 0.072; Across strains *r*-value: −0.86, *p*-value: 0.0001*; *significant *p*-values.

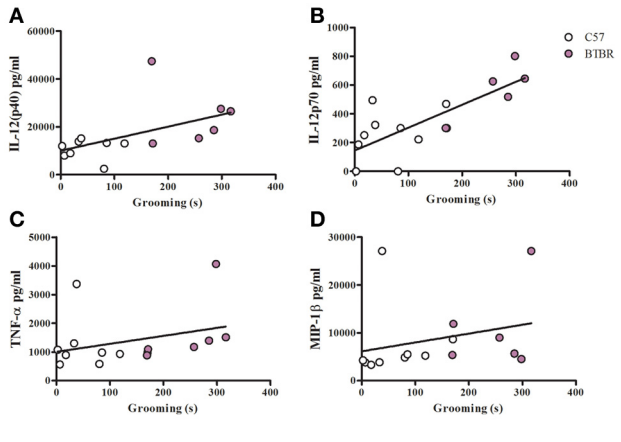


FIGURE 6 | Grooming correlation in IFNγ/LPS stimulated macrophages. Scatter graphs and linear regression of IL-12(p40) **(A)**, IL-12(p70) **(B)**, TNFα **(C)**, and MIP-1β **(D)** in IFNγ/LPS stimulated macrophages. Correlation coefficients (*r*-values) and *p*-values were calculated with Spearman's correlation analysis. **(A)** C57 *r*-value: 0.26, *p*-value: 0.536; BTBR *r*-value: 0.09, *p*-value: 0.919; Across strains *r*-value: 0.72, *p*-value: 0.004* **(B)** C57 *r*-value: 0.39, *p*-value: 0.291; BTBR *r*-value: 0.87, *p*-value: 0.033*; Across strains *r*-value: 0.77, *p*-value: 0.001* **(C)** C57 *r*-value: 0.02, *p*-value: 0.966; BTBR *r*-value: 0.94, *p*-value: 0.005*; Across strains *r*-value 0.51, *p*-value: 0.005* **(D)** C57 *r*-value: 0.70, *p*-value: 0.036*; BTBR *r*-value: 0.20, *p*-value: 0.704; Across strains *r*- value: 0.63, *p*-value: 0.011*; *significant *p*-values.

In this study, we have compared two inbred strains, which were selected for their possession of autism-relevant features or lack thereof (BTBR and C57, respectively) (McFarlane et al., 2008). Given the potential genetic differences between these

two inbred strains, it would be possible that these observed immunological differences are circumstantial and unrelated to behavior. To better understand the relationship between autism-like behavior and immune phenotype, we measured social and repetitive behavior in each mouse, and analyzed the correlation between these behavioral measurements and cytokine profiles. We observed only one cytokine correlation with social behavior (**Figure 2**), which may suggest that social behavior is not heavily affected by immune function within BTBR mice as a group.

Although we did not see strong correlations between social behavior and immune measures in these experiments, the inflammation in BTBR mice may have an impact on fetal development *in-utero*. Maternal immune activation (MIA) in C57 mice results in elevated expression of IL-6, IL-12(p40), IL-12(p70), IL-13, IL-15, IFNγ, TNFα, and IL-10 in pregnant dams (Arrode-Bruses and Bruses, 2012), and leads to deficits in sociability in their offspring (Malkova et al., 2012). The data here-in suggest that BTBR mice innately produce higher levels of many of these cytokines, which may suggest that the maternal environment in which BTBR embryos are exposed to naturally resembles that of the MIA C57 model in terms of increased inflammatory cytokine exposure *in-utero*. Potentially, social impairment is more closely associated with prenatal exposures, while repetitive grooming seems to be related to ongoing elevated inflammation. Additional research, such as prenatal cross-fostering, may help differentiate the roles of the maternal BTBR environment and adult immune profiles in the development of autism-like symptoms in the BTBR mice.

Unlike the social behavior, repetitive grooming behavior correlated with a number of cytokine profiles under all tested culture conditions. IL-12(p40) correlated with increasing grooming behavior of BTBR mice in unstimulated macrophages, and this correlation was also observed across the two strains (**Figure 3**). Contrary to the positive correlation between IL-12(p40) and repetitive behavior, macrophage derived IL-10 was negatively associated with repetitive grooming across strains in both LPS stimulated and IL-4/LPS stimulated conditions (**Figures 4**, **5**), suggesting a relationship between M1/M2 polarization and repetitive behavior such that M1 was associated with more impairment and M2 with less grooming behaviors. Moreover, both IL-12(p40) and IL-12(p70) showed positive correlations with repetitive grooming post IFNγ/LPS exposure, further supporting a relationship between M1 macrophage cytokines and repetitive grooming.

Of note, a difference in IL-12(p40) was observed between BTBR and C57 strains. We initially measured IL-12(p40) using an allele specific capture antibody, which recognizes several common strains such as C57, BALB/c, and CH3 mice, but does not recognize IL-12(p40) in autoimmune prone strains such as NOD or SJL strains of mice (Ymer et al., 2002). IL-12(p40) from BTBR was also undetectable with this antibody, but was detectable with a non-allele specific anti-IL-12(p40) capture antibody (data not shown). Although we did not test for IL-12(p40) allelic differences between these two strains, an allelic difference may indicate a functional difference for IL-12(p40) *in-vivo.* Interestingly, IL-10 production is also higher in BTBR mice. The synergistic effect of functionally abnormal IL-12p40 allele and high levels of IL-10 production in response to IFNγ may help explain how BTBR mice can both express higher IL-12(p40) and IL-12(p70) than their C57 counterparts, but also be more susceptible to Listeriosis (Heo et al., 2011).

The relationship between repetitive grooming and immune function, particularly in myeloid cells, has been recently illustrated by research in the Hoxb8 deficient mice. Hoxb8 is a myeloid expressed gene associated with repetitive pathological grooming in mice deficient for the gene and excessive grooming is reduced with wild-type bone marrow transplant (Chen et al., 2010). Given this data suggesting that peripherally derived immune cells can have a profound effect on normal grooming behavior, it is possible that ongoing myeloid inflammation may be related to repetitive grooming behavior in animal models. Further evidence that peripherally derived hematopoietic cells can improve behavior was illustrated in a recent paper demonstrating that bone marrow transplant arrested disease development in $MeCP2^{+/-}$ mouse models of Rett's syndrome (Derecki et al., 2012).

Research in children with autism also implicates the role of myeloid cells in the pathology of the disorder. Immunohistochemistry of postmortem brain samples have revealed elevated numbers of microglia in brain parenchyma, and increased perivascular macrophages as well as elevated microglial and perivascular macrophage activation in the brains of children with autism, and increased levels of MCP-1 (Vargas et al., 2005; Morgan et al., 2010). Consistent with findings in the brain, studies examining peripheral myeloid function have revealed increased numbers of circulatory monocytes in the blood and increased cytokine production following TLR4 stimulation in monocytes of children with autism including increased levels of IL-1β, IL-6, and IL-23 and associations with behavioral assessment scores (Sweeten et al., 2003; Jyonouchi et al., 2008; Enstrom et al., 2010). Similar to findings in microglia and monocytes, there is also evidence of atypical distribution of dendritic cell populations in children with autism (Breece et al., 2013), suggesting that many branches of the myeloid system are affected in the disorder. The data described here-in draws many parallels between previously recorded immune phenomena in humans, and the immune profile of the BTBR mouse and warrants a closer examination of neuro-immune interactions in the development of autism spectrum disorders.

In this study, we have demonstrated an inflammatory immune profile is displayed in the asocial BTBR mouse strain as well as associations between inflammation and M1 associated cytokines with repetitive grooming behavior. The immune phenotype of BTBR mice draws several parallels to observation in children with autism particularly high IL-12, IL-6, and MCP-1 production observed in both children with autism and BTBR mice. Although there are caveats in comparing the immunophenotype of two different strains based on behavior, the observed correlations between autism-relevant behaviors and immunological measures suggest the inflammatory phenotype of the BTBR mouse is more than circumstantial. Together these data suggests that the BTBR mouse may possess more than a behavioral similarity to autism

in humans, but may also share some physiological symptoms associated with the disorder as well. Although it may not be possible to find a true animal model for autism, the BTBR mouse may prove to be a useful model to study the relationship between inflammation and behavior, the fruits of which may one day be applied to autism and other inflammatory disorders that manifest in behavioral deficits.

ACKNOWLEDGMENTS

This work was supported by NIH T32MH073124, Jane Botsford Johnson Foundation, Peter Emch Foundation, Barbara and Michael Bass Foundation, and the Brain & Behavior Research Foundation (formerly known as National Alliance for Research on Schizophrenia and Depression). The authors would like to thank Duy Pham for his technical support.

REFERENCES

Abrahams, B. S., and Geschwind, D. H. (2008). Advances in autism genetics: on the threshold of a new neurobiology. *Nat. Rev. Genet.* 9, 341–355. doi: 10.1038/nrg2346

APA. (2000). *Diagnostic and Statistical Manual of Mental Disorders*. Arlington, VA: American Psychiatric Publishing Inc.

Arrode-Bruses, G., and Bruses, J. L. (2012). Maternal immune activation by poly(I:C) induces expression of cytokines IL-1beta and IL-13, chemokine MCP-1 and colony stimulating factor VEGF in fetal mouse brain. *J. Neuroinflammation* 9, 83. doi: 10.1186/1742-2094-9-83

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I. N., and Van de Water, J. (2011a). Altered T cell responses in children with autism. *Brain Behav. Immun.* 25, 840–849. doi: 10.1016/j.bbi.2010.09.002

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I., and Van de Water, J. (2011b). Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain Behav. Immun.* 25, 40–45. doi: 10.1016/j.bbi.2010.08.003

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I. N., and Van de Water, J. (2011c). Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *J. Neuroimmunol.* 232, 196–199. doi: 10.1016/j.jneuroim.2010.10.025

Ashwood, P., Wills, S., and Van de Water, J. (2006). The immune response in autism: a new frontier for autism research. *J. Leukoc. Biol.* 80, 1–15. doi: 10.1189/jlb.1205707

Benoit, M., Desnues, B., and Mege, J. L. (2008). Macrophage polarization in bacterial infections. *J. Immunol.* 181, 3733–3739.

Breece, E., Paciotti, B., Nordahl, C. W., Ozonoff, S., Van de Water, J. A., Rogers, S. J., et al. (2013). Myeloid dendritic cells frequencies are increased in children with autism spectrum disorder and associated with amygdala volume and repetitive behaviors. *Brain Behav. Immun.* 31, 69–75. doi: 10.1016/j.bbi.2012.10.006

Chadman, K. K., Gong, S., Scattoni, M. L., Boltuck, S. E., Gandhy, S. U., Heintz, N., et al. (2008). Minimal aberrant behavioral phenotypes of neuroligin-3 R451C knockin mice. *Autism Res.* 1, 147–158. doi: 10.1002/aur.22

Chen, S. K., Tvrdik, P., Peden, E., Cho, S., Wu, S., Spangrude, G., et al. (2010). Hematopoietic origin of pathological grooming in Hoxb8 mutant mice. *Cell* 141, 775–785. doi: 10.1016/j.cell.2010.03.055

Crawley, J. N. (2007). Mouse behavioral assays relevant to the symptoms of autism. *Brain Pathol.* 17, 448–459. doi: 10.1111/j.1750-3639.2007.00096.x

Derecki, N. C., Cronk, J. C., Lu, Z., Xu, E., Abbott, S. B., Guyenet, P. G., et al. (2012). Wild-type microglia arrest pathology in a mouse model of Rett syndrome. *Nature* 484, 105–109. doi: 10.1038/nature10907

Derecki, N. C., Privman, E., and Kipnis, J. (2010a). Rett syndrome and other autism spectrum disorders–brain diseases of immune malfunction? *Mol. Psychiatry* 15, 355–363. doi: 10.1038/mp.2010.21

Derecki, N. C., Cardani, A. N., Yang, C. H., Quinnies, K. M., Crihfield, A., Lynch, K. R., et al. (2010b). Regulation of learning and memory by meningeal immunity: a key role for IL-4. *J. Exp. Med.* 207, 1067–1080. doi: 10.1084/jem.20091419

Derecki, N. C., Quinnies, K. M., and Kipnis, J. (2011). Alternatively activated myeloid (M2) cells enhance cognitive function in immune compromised mice. *Brain Behav. Immun.* 25, 379–385. doi: 10.1016/j.bbi.2010.11.009

Enstrom, A. M., Onore, C. E., Van de Water, J. A., and Ashwood, P. (2010). Differential monocyte responses to TLR ligands in children with autism spectrum disorders. *Brain Behav. Immun.* 24, 64–71. doi: 10.1016/j.bbi.2009.08.001

Heo, Y., Zhang, Y., Gao, D., Miller, V. M., and Lawrence, D. A. (2011). Aberrant immune responses in a mouse with behavioral disorders. *PLoS ONE* 6:e20912. doi: 10.1371/journal.pone.0020912

Jouanguy, E., Doffinger, R., Dupuis, S., Pallier, A., Altare, F., and Casanova, J. L. (1999). IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Curr. Opin. Immunol.* 11, 346–351. doi: 10.1016/S0952-7915(99)80055-7

Jyonouchi, H., Geng, L., Cushing-Ruby, A., and Quraishi, H. (2008). Impact of innate immunity in a subset of children with autism spectrum disorders: a case control study. *J. Neuroinflammation* 5, 52. doi: 10.1186/1742-2094-5-52

Li, X., Chauhan, A., Sheikh, A. M., Patil, S., Chauhan, V., Li, X. M., et al. (2009). Elevated immune response in the brain of autistic patients. *J. Neuroimmunol.* 207, 111–116. doi: 10.1016/j.jneuroim.2008.12.002

Lintas, C., Sacco, R., and Persico, A. M. (2012). Genome-wide expression studies in autism spectrum disorder, Rett syndrome, and Down syndrome. *Neurobiol. Dis.* 45, 57–68. doi: 10.1016/j.nbd.2010.11.010

Malkova, N. V., Yu, C. Z., Hsiao, E. Y., Moore, M. J., and Patterson, P. H. (2012). Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain Behav. Immun.* 26, 607–616. doi: 10.1016/j.bbi.2012.01.011

Mantovani, A., Sica, A., and Locati, M. (2005). Macrophage polarization comes of age. *Immunity* 23, 344–346. doi: 10.1016/j.immuni.2005.10.001

McFarlane, H. G., Kusek, G. K., Yang, M., Phoenix, J. L., Bolivar, V. J., and Crawley, J. N. (2008). Autism-like behavioral phenotypes in BTBR T+tf/J mice. *Genes Brain Behav.* 7, 152–163. doi: 10.1111/j.1601-183X.2007.00330.x

Mikita, J., Dubourdieu-Cassagno, N., Deloire, M. S., Vekris, A., Biran, M., Raffard, G., et al. (2011). Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. *Mult. Scler.* 17, 2–15. doi: 10.1177/1352458510379243

Morgan, J. T., Chana, G., Pardo, C. A., Achim, C., Semendeferi, K., Buckwalter, J., et al. (2010). Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism. *Biol. Psychiatry* 68, 368–376. doi: 10.1016/j.biopsych.2010.05.024

Mosser, D. M., and Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–969. doi: 10.1038/nri2448

Moy, S. S., Nadler, J. J., Young, N. B., Nonneman, R. J., Segall, S. K., Andrade, G. M., et al. (2008). Social approach and repetitive behavior in eleven inbred mouse strains. *Behav. Brain Res.* 191, 118–129. doi: 10.1016/j.bbr.2008.03.015

Moy, S. S., Nadler, J. J., Young, N. B., Perez, A., Holloway, L. P., Barbaro, R. P., et al. (2007). Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. *Behav. Brain Res.* 176, 4–20. doi: 10.1016/j.bbr.2006.07.030

Onore, C., Careaga, M., and Ashwood, P. (2012). The role of immune dysfunction in the pathophysiology of autism. *Brain Behav. Immun.* 26, 383–392. doi: 10.1016/j.bbi.2011.08.007

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., et al. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13, 715–725. doi: 10.1016/S1074-7613(00)00070-4

Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., et al. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* 73, 457–467. doi: 10.1016/0092-8674(93)90134-C

Rothman, K. J. (1990). No adjustments are needed for multiple comparisons. *Epidemiology* 1, 43–46. doi: 10.1097/00001648-199001000-00010

Scattoni, M. L., Gandhy, S. U., Ricceri, L., and Crawley, J. N. (2008). Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS ONE* 3:e3067. doi: 10.1371/journal.pone.0003067

Scattoni, M. L., Ricceri, L., and Crawley, J. N. (2011). Unusual repertoire of vocalizations in adult BTBR T+tf/J mice during three types of social encounters. *Genes Brain Behav.* 10, 44–56. doi: 10.1111/j.1601-183X.2010.00623.x

Silverman, J. L., Tolu, S. S., Barkan, C. L., and Crawley, J. N. (2010). Repetitive self-grooming behavior in the BTBR mouse model of autism is blocked by the mGluR5 antagonist MPEP. *Neuropsychopharmacology* 35, 976–989. doi: 10.1038/npp.2009.201

Silverman, J. L., Turner, S. M., Barkan, C. L., Tolu, S. S., Saxena, R., Hung, A. Y., et al. (2011). Sociability and motor functions in Shank1 mutant mice. *Brain Res.* 1380, 120–137. doi: 10.1016/j.brainres.2010.09.026

Sweeten, T. L., Posey, D. J., and McDougle, C. J. (2003). High blood monocyte counts and neopterin levels in children with autistic disorder. *Am. J. Psychiatry* 160, 1691–1693. doi: 10.1176/appi.ajp.160.9.1691

Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W., and Pardo, C. A. (2005). Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann. Neurol.* 57, 67–81. doi: 10.1002/ana.20315

Voineagu, I., Wang, X., Johnston, P., Lowe, J. K., Tian, Y., Horvath, S., et al. (2011). Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature* 474, 380–384. doi: 10.1038/nature10110

Yang, M., Clarke, A. M., and Crawley, J. N. (2009). Postnatal lesion evidence against a primary role for the corpus callosum in mouse sociability. *Eur. J. Neurosci.* 29, 1663–1677. doi: 10.1111/j.1460-9568.2009.06714.x

Yang, M., Perry, K., Weber, M. D., Katz, A. M., and Crawley, J. N. (2011a). Social peers rescue autism-relevant sociability deficits in adolescent mice. *Autism Res.* 4, 17–27. doi: 10.1002/aur.163

Yang, M., Silverman, J. L., and Crawley, J. N. (2011b). Automated three-chambered social approach task for mice. *Curr. Protoc. Neurosci.* Chapter 8, Unit 8.26. doi: 10.1002/0471142301.ns0826s56

Yang, M., Zhodzishsky, V., and Crawley, J. N. (2007). Social deficits in BTBR T+tf/J mice are unchanged by cross-fostering with C57BL/6J mothers. *Int. J. Dev. Neurosci.* 25, 515–521. doi: 10.1016/j.ijdevneu.2007.09.008

Ymer, S. I., Huang, D., Penna, G., Gregori, S., Branson, K., Adorini, L., et al. (2002). Polymorphisms in the Il12b gene affect structure and expression of IL-12 in NOD and other autoimmune-prone mouse strains. *Genes Immun.* 3, 151–157. doi: 10.1038/sj.gene.6363849

Yrigollen, C. M., Han, S. S., Kochetkova, A., Babitz, T., Chang, J. T., Volkmar, F. R., et al. (2008). Genes controlling affiliative behavior as candidate genes for autism. *Biol. Psychiatry* 63, 911–916. doi: 10.1016/j.biopsych.2007.11.015

Zhang, X., Goncalves, R., and Mosser, D. M. (2008). The isolation and characterization of murine macrophages. *Curr. Protoc. Immunol.* Chapter 14, Unit 14.1. doi: 10.1002/0471142735.im1401s83

Zou, H., Yu, Y., Sheikh, A. M., Malik, M., Yang, K., Wen, G., et al. (2011). Association of upregulated Ras/Raf/ERK1/2 signaling with autism. *Genes Brain Behav.* 10, 615–624. doi: 10.1111/j.1601-183X.2011.00702.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 July 2013; accepted: 16 August 2013; published online: 17 September 2013.

Citation: Onore CE, Careaga M, Babineau BA, Schwartzer JJ, Berman RF and Ashwood P (2013) Inflammatory macrophage phenotype in BTBR T+tf/J mice. Front. Neurosci. 7:158. doi: 10.3389/fnins.2013.00158

This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Neuroscience.