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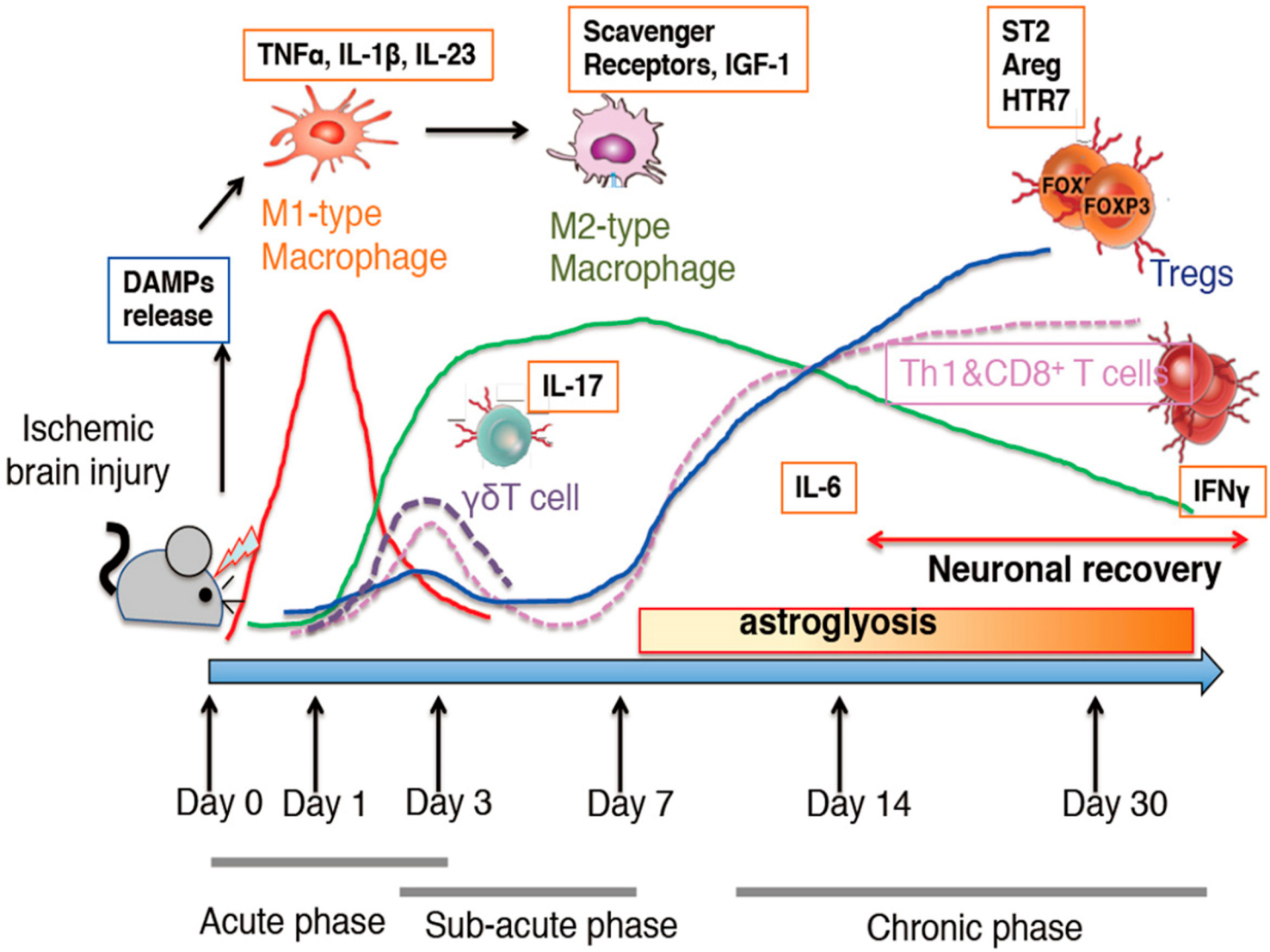

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Neuroimmunology and Neuroinflammation

CONTENTS

1 **Cerebrospinal fluid biomarkers for cognitive disorders. An introductory overview**
George P. Paraskevas
Neuroimmunol Neuroinflammation 2020;7:183-93. http://dx.doi.org/10.20517/2347-8659.2019.008

2 **Defining activation states of microglia in human brain tissue: an unresolved issue for Alzheimer's disease**
Douglas G. Walker
Neuroimmunol Neuroinflammation 2020;7:194-214. http://dx.doi.org/10.20517/2347-8659.2020.09

3 **Microbiome meets microglia in neuroinflammation and neurological disorders**
Rachel E. N. Reyes, Zeyu Zhang, Lei Gao, Liana Asatryan
Neuroimmunol Neuroinflammation 2020;7:215-33. http://dx.doi.org/10.20517/2347-8659.2020.13

4 **Microglial contributions to aberrant neurogenesis and pathophysiology of epilepsy**
Tanya R. Victor, Stella E. Tsirka
Neuroimmunol Neuroinflammation 2020;7:234-47. http://dx.doi.org/10.20517/2347-8659.2020.02

5 **Microglial heterogeneity: distinct cell types or differential functional adaptation?**
Savannah D. Benusa, Nicholas M. George, Jeffrey L. Dupree
Neuroimmunol Neuroinflammation 2020;7:248-63. http://dx.doi.org/10.20517/2347-8659.2020.03

6 **Resolution of inflammation and repair after ischemic brain injury**
Akihiko Yoshimura, Minako Ito
Neuroimmunol Neuroinflammation 2020;7:264-76. http://dx.doi.org/10.20517/2347-8659.2020.22

7 **The immune regulation of PD-1/PDL-1 axis, a potential biomarker in multiple sclerosis**
Maria Teresa Cencioni
Neuroimmunol Neuroinflammation 2020;7:277-90. http://dx.doi.org/10.20517/2347-8659.2020.18

8 **What is the role of Brain derived neurotrophic factor in Multiple Sclerosis neuroinflammation?**
Viviana Nociti
Neuroimmunol Neuroinflammation 2020;7:291-9. http://dx.doi.org/10.20517/2347-8659.2020.25

Paraskevas. *Neuroimmunol Neuroinflammation* 2020;7:183-93
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Neuroimmunology and Neuroinflammation

Review

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Cerebrospinal fluid biomarkers for cognitive disorders. An introductory overview

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Abstract

The core (established) cerebrospinal fluid biomarkers of Alzheimer's disease (AD), namely amyloid-beta peptide, total tau protein and phospho-tau protein, have become a part of the diagnostic workup of patients with cognitive disorders in many specialized centers, especially for ambiguous cases. Combined, these biomarkers can identify the presence or absence of an AD biochemical process with sensitivities and specificities approaching or exceeding 90% in both dementia and pre-dementia stages of AD. Thus, they have been incorporated in various sets of research or clinical diagnostic criteria and recommendations. Results that are atypical, incompatible with AD, or inconclusive may occur, necessitating the use of other cerebrospinal fluid or imaging biomarkers.

Keywords: Cerebrospinal fluid, tau, phospho-tau, amyloid-beta, Alzheimer's disease, alpha-synuclein, TDP-43, neurofilament light protein

INTRODUCTION

Almost 25 years after their first introduction, cerebrospinal fluid (CSF) biomarkers have become a part of the diagnostic workup of patients with cognitive disorders in many specialized centers. Furthermore, they provide neurochemical information about the disorder underlying each individual patient's clinical presentation, which currently should be viewed as a biological process, sometimes starting many years prior to symptom onset and gradually evolving into a typical or atypical clinical phenotype. This paper provides an introductory, concise review, regarding the current status and future perspectives of CSF biomarker use.

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WHY DO WE NEED BIOMARKERS?

Alzheimer's disease (AD) is the most common type of dementia[1], followed by vascular cognitive impairment (VCI)[2], dementia with Lewy bodies (DLB)[3], frontotemporal dementia (FTD)[4] and others. Until relatively recently, diagnosis of AD was made according to clinically based criteria[5]. These criteria may show high diagnostic accuracy, especially when typical cases are examined in specialized centers[6]. However, it is long known that in the community, in early, presenile or atypical cases and in the presence of comorbidities, diagnostic accuracy may drop substantially, with clinicopathological concordance rates sometimes as low as 62.5%[7-9]. Furthermore, it is now recognized that AD, typically presenting as an amnestic dementia syndrome, may rarely have frontal (sometimes frontotemporal-like)[10,11], posterior[10,11], language[10-12] and even corticobasal-like presentations[13,14]. Thus, the same disease may present with different phenotypes, and one phenotype may be caused by different diseases/pathologies. Mixed pathologies are not infrequent in senile cases[15], especially AD mixed with various types of vascular lesions[16], or DLB with concomitant AD pathology[17]. Such mixed pathologies may modify the clinical presentation[18,19] and the rate of disease progression[20]. In addition, some patients present very early, in a symptomatic but pre-dementia stage [mild cognitive impairment (MCI) and MCI due to AD][21]. On the other hand, when the clinical impression is against AD, there is still a 39% chance for pathological verification of AD (co)existence[22].

The above are not uncommon causes of diagnostic confusion in everyday practice. Of course, the gold standard for diagnostic verification is *post mortem* pathological examination. However, correct *ante mortem* diagnosis is necessary since it may help in predicting prognosis and it is likely to affect therapeutic decisions[23]. Thus, biomarkers are needed to serve as objective diagnostic tools during life. In the last 3 decades, various biomarkers have been developed (some being incorporated in various sets of diagnostic criteria), including structural neuroimaging (pattern of atrophy as a marker of neuronal injury), functional neuroimaging with positron emission tomography (PET), either as FDG-PET (hypometabolism as a marker of neuronal injury) or PET at least for amyloid-beta (Aβ), and CSF biomarkers[10,11]. The last have probably received the most attention.

ESTABLISHED (CORE) CSF BIOMARKERS OF AD

In an oversimplified scheme, there are two biochemical processes and pathological hallmarks of AD: (1) misfolding, oligomerization and finally polymerization and extracellular aggregation of Aβ, in the form of amyloid plaques, and (2) intracellular hyperphosphorylation and polymerization of the microtubule-associated protein tau, forming paired helical filaments which in turn aggregate in the form of neurofibrillary tangles[24,25]. The former process mobilizes various mechanisms that are toxic to neurons[26], and the second results in destabilization of microtubules and dysfunction of the cytoskeleton and of axonal transport[27]. Both processes, acting synergistically, lead to neuritic, synaptic and neuronal loss, through a vicious circle of interconnecting final pathways of oxidative stress, excitotoxicity, mitochondrial dysfunction, apoptosis and Ca^{2+}-mediated cell death[28-30], while prion-like spread[31] and neuroinflammation[32-34] are increasingly recognized as important early mechanisms.

Total tau protein (τ_T)[35], hyperphosphorylated tau, especially at a threonine residue at position 181 ($\tau_{P\text{-}181}$)[36] and Aβ peptide with 42 amino acids ($A\beta_{42}$)[37] can be quantified in the CSF. In AD, τ_T is increased, and traditionally, this is viewed as a marker of neuronal/axonal injury[38]; $\tau_{P\text{-}181}$ is also increased and this is considered a more specific marker of tangle formation[39]. On the other hand, $A\beta_{42}$ is decreased and this is considered (inversely) a marker of amyloid burden[40]. The above markers are useful in the discrimination of AD from normal aging and other dementias, and even abnormal τ_T alone may show high sensitivity and, in a few diagnostic questions, adequate specificity for the diagnosis of AD[41]. Combinations of the above biomarkers in the form of various formulas (including the Hulstaert formula[42]) or ratios (including $\tau_T/A\beta_{42}$ or $\tau_{P\text{-}181}/A\beta_{42}$[43,44]) further increase their diagnostic value.

HOW EARLY DO THE CLASSICAL BIOMARKERS BECOME ABNORMAL?

Currently, AD is viewed as a pathological or neurobiological entity, characterized by a continuum of 3 stages, starting as a preclinical ("asymptomatic at risk" or "presymptomatic") stage, which later on progresses to a symptomatic but pre-dementia stage (MCI) and finally to the dementia stage[11,45]. It seems that in most cases CSF biomarkers become abnormal during the preclinical stage of AD[45], and on the basis of studies in families with autosomal dominant AD, this may occur even 10-20 years prior to the expected age of symptom onset[46]. In patients with MCI, abnormalities are detected 5-10 years before progression to dementia[47]. Usually, the first abnormality detected is a decrease in $A\beta_{42}$, followed by an increase in τ_{P-181} and τ_T, but the reverse order may sometimes be observed[45]. Sometimes, only the $A\beta_{42}$ decrease is seen in the preclinical stage, and the increase in τ_{P-181} and τ_T is observed in the pre-dementia (MCI) stage of AD[48]. Thus, in the vast majority of patients, all 3 classical biomarkers are already abnormal when patients enter the dementia stage and in many (if not most), at the beginning of the MCI stage as well. CSF levels may continue to change during disease progression[46,48,49]. Such changes may be important from the neurochemical point of view, and it has been suggested that they may correlate with the stage of the disease[48]. However, from a diagnostic point of view, the changes compared to controls are small, and thus, these biomarkers are considered as state and not stage markers[49].

DEFINITION OF THE ALZHEIMER'S CLASSICAL CSF BIOMARKER PROFILE (SIGNATURE)

In the research diagnostic criteria for AD of the International Working Group (IWG-2), both decreased $A\beta_{42}$ and increased tau protein (either τ_T or τ_{P-181}) are considered as *in vivo* evidence of AD pathology, with sensitivities and specificities approaching or exceeding 90%[11]. However, more recent recommendations suggest that all 3 biomarkers should be abnormal[50]. Indeed, this may increase specificity, and abnormality of all 3 biomarkers is highly suggestive (and specific) of the presence of AD, while normal values of all 3 biomarkers is highly suggestive of the absence of AD pathology[50]. In patients with MCI, the combination of all 3 markers (τ_T and the $A\beta_{42}/\tau_{P-181}$ ratio) identified those harboring AD pathology with sensitivities and specificities of 95% and 87%, respectively[51].

In pathologically verified cases, the combination of $A\beta_{42}$ and τ_T identified AD patients, discriminating them from other dementias or controls with sensitivity and specificity of 90% and 89%, respectively[52], while the combination of $A\beta_{42}$ with the more specific τ_{P-181} discriminated AD from other dementias with sensitivity and specificity of 80%-88% and 93%-100%, respectively[52,53].

The above indicates that, ideally, the AD CSF biomarker signature should be defined as abnormal values of all 3 core biomarkers. However, the combination of $A\beta_{42}$ with one of the tau forms (either total or phosphorylated) may be sufficient in everyday practice.

ANSWERED AND UNANSWERED QUESTIONS

Classical AD biomarkers are useful in everyday practice since they can discriminate AD from normal aging[43] and psychiatric conditions[54]. They offer an added diagnostic value in everyday differential diagnosis of dementia patients, since they increase diagnostic confidence[41] and correctly identify the presence or absence of AD in 82% of patients with uncertain clinical diagnosis[55]. They can be useful in the differential diagnosis between AD and FTD[56], and they can identify the additional presence or absence of AD in patients with cerebrovascular disease and dementia[44], including subcortical small vessel disease[57]. These biomarkers may also determine the additional presence of AD in patients with DLB[58] and those with normal pressure hydrocephalus[59]. Additionally, they can identify the presence or absence of AD biochemical process in patients with certain cognitive and/or parkinsonian syndromes such as primary progressive aphasia[12], posterior cortical atrophy[60] and corticobasal syndrome[13].

Of course, CSF AD biomarkers are not standalone tools, and they should be used in conjunction with clinical, imaging, neuropsychological and other biochemical data to reach the correct diagnosis[11]. Keeping that in mind, CSF $A\beta_{42}$, τ_T and τ_{P-181} fulfill most of the criteria required for valid biomarkers[61], since they reflect key biochemical mechanisms of AD, and combined, they provide sensitivities and specificities greater than 80%-85%. Sampling needs lumbar puncture, which is less agreeable than urine or blood sampling. However, it is a minimally invasive procedure, usually well-tolerated and with a low incidence of post-lumbar puncture headache (< 4.5%) in dementia patients[43,62]. Thus, the 3 core CSF biomarkers were gradually incorporated in research and/or clinical diagnostic criteria for AD in the dementia (typical or atypical presentations)[10,11,63], MCI[11,64] and preclinical stages[65], and if testing is available, they are currently considered as part of the diagnostic workup of cognitive disorders, especially in ambiguous cases[66-68]. Since new disease-modifying or preventive treatments are currently underway, CSF biomarkers may be used for the selection of patients suitable for clinical trials across all stages of AD (including the preclinical stage) and/or for monitoring treatment effects[69,70].

With time, it has become evident that biomarkers can detect CSF signatures different from the one observed in AD. The term "suspected non-Alzheimer pathophysiology" (SNAP) was introduced for a biomarker profile with normal $A\beta_{42}$ but an abnormal marker of neuronal injury or neurodegeneration, while the term "primary age-related tauopathy" has been used for the tau pathology picture in the medial temporal lobe (hippocampus, entorhinal cortex), with or without minimal Aβ pathology[71]. In patients with normal $A\beta_{42}$, the $A\beta_{42}/A\beta_{40}$ ratio may be used to confirm the absence of amyloid abnormality, since it "corrects" observed $A\beta_{42}$ levels for the total level of $A\beta_{40}$ (the most abundant form of Aβ peptide)[67,72]. When amyloid normality is confirmed, AD becomes unlikely[50] and tauopathies, TDP-43 proteinopathies and other pathologies may be considered to explain SNAP cases[73]. Controversies and questions concerning the "non-AD" biomarker profiles and the underlying pathologies have led to a modification of the 2011 National Institute on Aging and Alzheimer's Association separate recommendations[10,64,65], to a unified biological definition of AD across all stages and incorporating the various possible biomarker profiles and disease categories (AD or non-AD)[74]. This incorporation of "extended" biomarker profiles in diagnostic recommendations, may prove useful in many atypical presentations, including patients resembling or even fulfilling criteria for AD, but without the expected AD CSF biomarker signature, although biomarker levels may remain conflicting in occasional patients.

Another profile which may be observed is characterized by abnormality (reduction) of only $A\beta_{42}$, while τ_T and τ_{P-181} being normal. In this case, the $A\beta_{42}/A\beta_{40}$ may be used to confirm or exclude amyloid abnormality. If amyloid reduction is confirmed, AD pathology may be less likely in patients with full-blown dementia, but it is still a possibility, especially in pre-dementia patients[50] and may be compatible with the "AD pathological change"[74]. This profile may also be observed in vascular cognitive decline[57] and in Lewy body synucleinopathies, including PD, PDD and especially DLB[75].

Furthermore, there is always the problem of mixed pathologies, especially in the elderly. In a patient with a clinical picture suggestive of DLB, the identification of the typical AD CSF signature, may indicate mixed synucleinopathy with concomitant AD pathology[11,76], but cases of AD with unusual DLB-like presentations have been described[77]. Even in the most common scenario of mixed pathology, the question arises as to whether it represents DLB with some degree of AD pathology, AD with some degree of Lewy-pathology or equally severe pathology of both types[58]. Similarly, in a patient with a FTD-like clinical picture, the identification of the typical AD CSF signature may serve as exclusion criterion for FTD[78], suggesting AD with an atypical clinical presentation (frontal variant)[11], but mixed pathology cannot be excluded, since patients with concomitant FTD and AD do exist[79].

Some patients may show borderline or gray-zone levels in one or more of the classical biomarkers. The $\tau_T/A\beta_{42}$ and $\tau_{P-181}/A\beta_{42}$ ratios may be of some help in such patients[12], but not always. The "Erlangen Score",

which depends on normal, border-zone or abnormal biomarker levels, has been suggested to determine the level of neurochemical probability for (or against) AD in both dementia[80] and pre-dementia stages[81].

In case of atypical, conflicting or inconclusive CSF biomarker results, other neurochemical and/or imaging biomarkers, and/or later repetition of CSF sampling and analysis may be necessary[50].

VARIABILITY OF BIOMARKER RESULTS

Despite intensive research, there is still a significant inter- and intra-laboratory variability in the results of biomarker level determination, as a result of pre-analytical, analytical, post-analytical and kit-related factors, even between laboratories using the same methods[82-86]. During the last decade, various international initiatives, quality control programs and international workshops have been organized to reduce variability and harmonize the levels of biomarkers[67,82,83], including the "Biomarkers for Alzheimer's disease and Parkinson's disease" project of the Joint Programming Neurodegenerative Disease (JPND-BIOMARKAPD)[87]. As a result, recommendations have been published regarding lumbar puncture, pre-analytical and analytical standardized operating procedures[82,88-90], leading to improvement in diagnostic performance and reduction of measurement errors[91]. Although a measurement error of ± 20% in only one of the three biomarkers may have a minimal effect on overall diagnostic performance in everyday practice (variability ≤ 8%), errors of greater magnitude and/or affecting more than one biomarker, may lead to a significant decrease in diagnostic accuracy[92]. Newer methods for the determination of classical biomarkers may show better repeatability and reproducibility and less inter-laboratory variability[66,93].

NEW AND EMERGING BIOMARKERS FOR AD AND OTHER DISORDERS

Among many molecules studied in AD, neurogranin, neurofilament light (NFL), the ectodomain of triggering receptor expressed on myeloid cells 2 (sTREM2) and visinin-like protein 1 (VILIP-1) may serve as markers of synaptic loss, neuronal/axonal damage, microglial activation and neurodegeneration, respectively[66-68].

Recently, promising results have been published for CSF TDP-43 in patients with FTD and/or amyotrophic lateral sclerosis (ALS)[94-96]. The $\tau_{P\text{-}181}/\tau_T$ ratio has been suggested as another marker, which may prove helpful in the identification of FTD pathology[97], but its combination with TDP-43 may increase its diagnostic value even more[95]. NFL may also have some value in patients with FTD and/or ALS[66]. Further studies are needed, and they are in progress, both for validation and standardization of TDP-43 methods and for identifying the optimum combination of TDP-43 with other biomarkers for *in vivo* detection of the FTD subtype.

Alpha-synuclein (α-syn) has been studied as a biomarker of Lewy body synucleinopathies, in the differential diagnosis of cognitive and/or movement disorders[98,99]. Several studies have revealed that in synucleinopathies such as DLB, CSF α-syn levels are reduced, as compared to controls or AD[100,101]. However, increased levels in DLB *vs.* AD[102] or PDD[103] have also been reported, especially of oligomeric α-syn[104], while for PD or PDD, a non-significant reduction was too small to achieve diagnostic significance *vs.* controls and other movement disorders[13] or AD[103]. The above discrepancies indicate that, despite intensive research, there are methodological problems in α-syn quantification. Determination of α-syn needs strict pre-analytical control for confounding factors (especially bloody CSF), while assay parameters such as antibodies used, and forms of α-syn detected, necessitate further studies before one or more robust tests become widely acceptable[99,105].

CONCLUDING REMARKS

Classical CSF biomarkers of AD are useful tools in the (differential) diagnosis of patients with cognitive decline, especially in early or atypical cases [Table 1]. They are useful in differentiating AD from normal

Table 1. Levels of classical cerebrospinal fluid Alzheimer's disease biomarkers in various cognitive disorders

	$A\beta_{42}$ or $A\beta_{42}/A\beta_{40}$	Total tau (τ_T)	Phospho-tau*
Alzheimer's disease	Decreased	Increased	Increased
Vascular cognitive impairment	May be decreased in some patients	May be increased in some patients	Normal
Frontotemporal dementia	May rarely be decreased	May be increased in some patients	May be increased in some patients
Dementia with Lewy bodies	Frequently decreased	May be increased in some patients	Normal
Creutzfeldt-Jakob disease	May be decreased in some patients	Extremely increased	Normal
Normal aging	Normal	Normal	Normal
Psychiatric disorders	Normal	Normal	Normal

Based on the references cited throughout the text. *Usually for $\tau_{P\text{-}181}$, others have also been suggested

aging, psychiatric disorders such as depression, pure vascular cognitive impairment, pure DLB and FTD, and they can identify atypical and misleading clinical presentations of AD, or the coexistence of AD in other primary (such as VCI or DLB) or secondary cognitive disorders[12,14,44,54,56-59]. However, they should always be used in combination with clinical, neuropsychological and imaging data[15], and due to variability of measurements, each laboratory should establish their own normal or cut-off values[66].

CSF biomarkers detect normal or abnormal biochemistry, offering (during life) an alternative to post-mortem pathology and showing a very good concordance with pathological diagnosis[66]. Thus, many, if not most, of patients can be correctly diagnosed. However, borderline or inconclusive results may occur in some patients, requiring repetition of measurements and/or use of additional biomarkers[50,96,102]. Furthermore, classical CSF biomarkers cannot accurately detect mixed degenerative pathologies, which are not unusual in older patients. For example, the identification of an AD biomarker profile in a patient with dementia, parkinsonism and hallucinations, may indicate an atypical clinical presentation of AD, AD with some additional Lewy bodies, DLB with some additional AD-type pathology, or a severe degree of both pathologies[58]. This further necessitates the use of additional biomarkers (in the above case, α-syn). Unfortunately, methodological issues requiring further investigation prevent some of the newer biomarkers such as α-syn and TDP-43 to be currently considered "established".

The 3 classical AD biomarkers (τ_T, $A\beta_{42}$ and $\tau_{P\text{-}181}$) become 4 by adding $A\beta_{40}$. Adding NFL, α-syn, TDP-43 and others increases the number to at least 7. Adding them to structural and functional neuroimaging and possibly to genetic biomarkers, leads to a tempting increase of available data for patients; unfortunately, there is an even more substantial increase in cost, while the diagnostic accuracy may not be equally increased in some patients. Instead of an "all for all" approach, a personalized, precision medicine approach may be more appropriate[106], while blood biomarkers may be adequate for some patients[107].

The ability to detect the AD CSF biochemical signature in pre-dementia and especially in pre-symptomatic subjects, raises some ethical issues[108]. Communication of a positive result in a non-demented subject may have adverse effects in quality of life and trigger significant emotional reactions[109]. Since the time of appearance of the initial vague symptom(s) is usually unpredictable, many authorities consider it inappropriate to perform such diagnostic tests in the majority of asymptomatic subjects (including families with autosomal dominant AD). However, other subjects prefer disclosure of the results, so that they can adjust their life accordingly (including measures for secondary prevention) or make important decisions before dementia affects their judgment[110]. Such parameters should be taken into consideration before determining CSF biomarkers and/or communicating results, especially in research settings[108].

On the other hand, early detection is important in correct classification of subjects in trials of disease-modifying approaches, which may be effective when given in pre-symptomatic stages of AD. Since such trials are usually multicenter, stability, robustness and harmonization of methods and results, regulatory guidance, operator training, quality control programs, strict adherence to recommendations for

standardized operating procedures and harmonization of diagnostic criteria used, as well as well-organized and secure patient data sharing, are all required and pose challenges that should be faced by specialized centers[111].

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

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Neuroimmunology and Neuroinflammation

Review

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Defining activation states of microglia in human brain tissue: an unresolved issue for Alzheimer's disease

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Abstract

The development of concepts concerning the role of microglia in different brain diseases has relied on studies of animal models or human brain tissue, which primarily use antibodies and immunohistochemistry techniques to make observations. Since initial studies defined increased expression of the major histocompatibility complex II protein human leukocyte antigen-DR as a means of identifying reactive, and therefore by implication, damage-causing microglia in Alzheimer's disease (AD) or Parkinson's disease (PD), understanding and describing their activation states has evolved to an unexpected complexity. It is still difficult to ascertain the specific functions of individual microglia, particularly those associated with pathological structures, using a narrow range of antigenic markers. As many approaches to developing treatments for AD or PD are focused on anti-inflammatory strategies, a more refined understanding of microglial function is needed. In recent years, gene expression studies of human and rodent microglia have attempted to add clarity to the issue by sub-classification of messenger RNA expression of cell-sorted microglia to identify disease-associated profiles from homeostatic functions. Ultimately all newly identified markers will need to be studied in situ in human brain tissue. This review will consider the gaps in knowledge between using traditional immunohistochemistry approaches with small groups of markers that can be defined with antibodies, and the findings from cell-sorted and single-cell RNA sequencing transcription profiles. There have been three approaches to studying microglia in tissue samples: using antigenic markers identified from studies of peripheral macrophages, studying proteins associated with altered genetic risk factors for disease, and studying microglial proteins identified from mRNA expression analyses from cell-sorting and gene profiling. The technical aspects of studying microglia in human brain samples, inherent issues of working with antibodies, and findings of a range of different functional microglial markers will be reviewed. In particular, we will consider

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markers of microglia with expression profiles that do not definitively fall into the pro-inflammatory or anti-inflammatory classification. These additional markers include triggering receptor expressed on myeloid cells-2, CD33 and progranulin, identified from genetic findings, colony stimulating factor-1 receptor, purinergic receptor P2RY12, CD68 and Toll-like receptors. Further directions will be considered for addressing crucial issues.

Keywords: Neuropathology, RNA-sequencing, TREM2, microglia, activation states, immunohistochemistry

INTRODUCTION

Alzheimer's disease (AD) and Parkinson's disease (PD) have become the most significant and feared brain diseases of elderly populations who are now enjoying longer lifespans due to more effective treatments for cancer, cardiovascular and metabolic diseases. AD is the most common cause of cognitive decline and dementia in elderly populations[1], while PD can lead to severe loss of mobility and independence, amongst other features[2]. Both diseases are significant causes of morbidity in elderly populations and share many common pathological features involving the accumulation of aggregated proteins. In AD, it is extracellular amyloid and neurofibrillary-associated phosphorylated tau neurites and tangles[3], while in PD, it is aggregated/phosphorylated alpha-synuclein accumulated into pathological inclusions[4]. These diseases are distinctive on account of the degenerative changes occurring in different brain regions; however, one common feature is the appearance of "activated" microglia within brain regions showing degenerative changes. Inflammation has become one of the targets being investigated as treatment strategies for these diseases, and the importance of studying microglia in relation to many different brain diseases is widely appreciated[5-7].

Initial antibody-based observations on "activated" microglia in postmortem brain tissues were made 30 years ago and gave rise to the inflammatory hypotheses for neurodegeneration. This is illustrated in Figure 1 and suggests that initial cell death or accumulation of aberrant/aggregated proteins [amyloid beta (Aβ), tau or alpha-synuclein (α-syn)] results in proinflammatory activation of microglia, causing the production of toxic and/or inflammatory cytokines. The resulting neurotoxicity would then accelerate further inflammation, thus exacerbating the neurodegenerative process. These concepts developed in the 80's and 90's might now be considered imprecise based on more recent findings. However, it was from this hypothesis that treatments for AD and PD with anti-inflammatory agents were developed and tested. This approach was supported by data from epidemiological studies that patients who had long-term usage of anti-inflammatory drugs for inflammatory conditions such as arthritis, had less dementia, which appeared to support the inflammatory hypothesis of AD[8]. However, although many anti-inflammatory compounds and strategies have proven effective in AD animal models, clinical trials of AD patients have generally shown no significant effect[9]. The purpose of this review is to consider the approaches used to define changes in microglial phenotypes and neuroinflammation in brain tissue, and discuss how the role of microglia in neurodegeneration should be considered in light of a wider range of markers identified from recent transcriptional profiling of microglia. The focus of this review will be on studies relating to AD, but many of the concepts might be applicable to PD, multiple sclerosis (MS) or stroke. The aim for this review is to bridge the gap between the studies that have analyzed transcription in large numbers of samples or isolated cells with *in situ* studies in human brain samples with antibodies to define the microenvironments of microglia in the specialized neuroanatomy of the human brain[10-12]. Ultimately, the best way to define the microglia responsible for damaging inflammation in brain samples will be with a single or small panel of markers that can be studied reliably in widely available types of pathological brain samples.

METHODOLOGY FOR INVESTIGATING MICROGLIAL PHENOTYPES

Antibody-based methodology for *in situ* localization of microglial antigens

Pioneering observations on microglia by Rio-Hortega[13] used traditional metal-based histological stains to

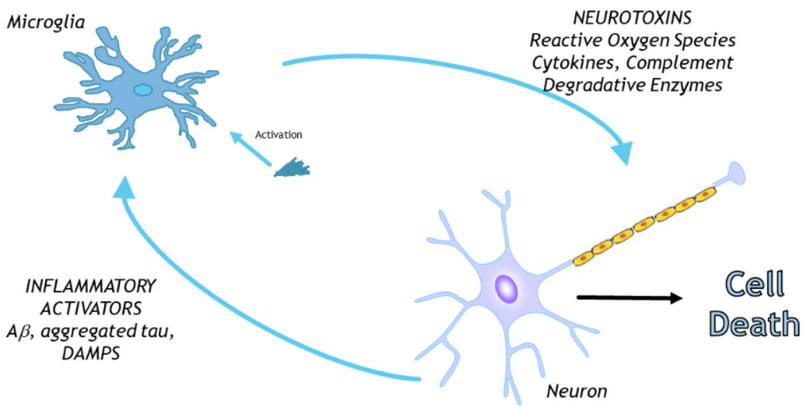

Figure 1. Hypothesis on the involvement of microglia in neurodegeneration

identify these cells. The fascinating history of their discovery has been reviewed by Tremblay *et al.*[14], but it was the use of specific antibodies in more recent times to sensitively identify microglia in human brain samples that re-launched this field of study. Studies by McGeer and colleagues employed an antibody to the major histocompatibility complex class II (MHCII) protein human leukocyte antigen-DR to identify what was described as "activated" or "reactive" microglia in AD and PD brain samples[15-18]. These types of microglia were enhanced around AD and PD pathological structures. Similar observations were made by Rogers and colleagues using the same marker[19]. Early studies highlighted recurring issues in the study of microglia in human autopsy tissue, namely with the antibodies used and the fixation methods of brain tissue samples for study[20]. Many microglial antigens, including human leukocyte antigen (HLA)-DR, are membrane-associated glycoproteins that are sensitive to tissue fixation with cross-linking fixatives such as paraformaldehyde/formalin and glutaraldehyde. The most widely available tissue samples for research are those taken for routine pathological examination and diagnosis at autopsy and usually involve long-term immersion fixation and paraffin-embedding using treatment with alcohols, xylene and similar solvents. Many validated monoclonal antibodies to macrophage/microglia antigens will not recognize them in tissue fixed in this manner, though a newer generation of antibodies, particularly monoclonal antibodies developed in rabbits, work more effectively when combined with different antigen-retrieval techniques[21]. Optimally-fixed tissue is tissue with short postmortem interval between death of donor subject and start of fixation, and a short period of fixation (48 h) of sliced brain coronal sections (not whole hemispheres) in buffered formalin/paraformaldehyde followed by preservation at 4 °C or -20 °C in an anti-freeze solution. Tissue preserved in this manner, which is then sectioned and processed for immunohistochemistry without paraffin embedding, has given optimal results for this investigator for detecting a number of different microglial proteins *in situ*[22,23].

Cell-sorting, nuclei-sorting, transcriptional profiling of inflammation and microglia

Expression profiling methods used to address this question have evolved rapidly over the last few years with RNA-sequencing becoming the predominant method of identification and quantification of genes expressed. There are now enormous amounts of data available online for carrying out analyses using various statistical criteria to identify the interactions of expressed microglial genes. For a more detailed understanding of these analytical approaches, the review of Chew and Petretto provides an overview of the different analytical approaches focusing on how the identification of transcriptional networks of microglia in AD can give insight into disease pathogenesis[10]. One observation by these authors was the lack of agreement between studies on which genes/markers should be the targets for tissue validation.

The findings from a number of RNA sequencing experiments of batch-sorted or single cell microglia isolated from AD or immune-stimulated animal models or human brain tissues will be considered. The

results have been analyzed comparing AD animals with non-transgenic control animals, or between AD and controls from aged human tissues[24]. Although the focus of this article is on understanding how to define activation states in human brains, findings from rodent models in this context have to be considered. Microglia can be directly isolated from the mouse brain by Dounce homogenization to break up tissue, filtering through 70 μm mesh, separation by magnetic beads conjugated with anti-myelin antibody to remove myelin, and then selected using a fluorescence-activated cell sorter with appropriate labeled antibodies (e.g., CD11b, CD45) to isolate immune cells, including microglia[25]. This basic approach will isolate populations of cells that can be diluted to allow the isolation of single cells or analysis in bulk. Refinements to these techniques have allowed the sorting and RNA profiling of cellular nuclei from frozen human and animal tissue samples[11,26]. Different approaches for microglial profiling are illustrated in Figure 2.

Using these approaches, it was shown that trans-membrane protein 119 (TMEM119) was a specific marker for microglia in mouse and human brains[25]. The isolation of microglia from human brains using the same methodology is possible but has some limitations. Human brain tissue is not usually amenable to Dounce homogenization and requires additional enzymatic digestion to dissociate tissue into single cells, and density gradient centrifugation to separate the myelin content from the cellular components[27]. Studies using these approaches aimed to define genes that are unique to microglia and not expressed, or expressed at low levels in blood monocytes/macrophages[28-31].

Recent important studies relating to inflammatory changes in AD brains identified a type of "disease-associated microglia" (DAM) that appear to be associated primarily with preventing inflammatory pathology rather than enhancing it[26,32,33]. Another key study involved the meta-analyses of multiple different gene profiling studies related to brain inflammation[24]. Amongst other findings, these studies confirmed TMEM119, purinergic receptor P2YR12 and fractalkine receptor CX3CR1 as markers with highly enriched expression in microglia compared to monocytes/macrophages.

DAM

The identification of DAM was primarily carried out using single cell microglia RNA sequencing in AD model mice (5xFAD) of different ages followed by validation in human tissue samples. The progressive changes in microglia phenotypes was from homeostatic (non-activated) to stage 1 DAM, which represents a state of proinflammatory activation, to stage 2 DAM, an altered phenotype that restricts neurodegenerative changes. Based on results that included the use of mice that are gene deficient (knockout) for the crucial triggering receptor expressed on myeloid cells (TREM2), it was shown that the transition from stage 1 to stage 2 DAM was dependent on Trem2 signaling. These data have implied that the activation of Trem2 signaling was protective rather than pathogenic[32]. [Gene identification primarily from studies using rodents will use the lower case abbreviation; genes primarily identified in human will use the upper-case abbreviation]. This study defined expression of genes Hexb (Beta-hexosaminidase subunit beta), Cst3 (Cystatin C), Cx3cr1 (Fractalkine receptor), Ctsd (Cathepsin D), Csf1r (Colony stimulating factor-1 receptor), Ctss (Cathepsin S), Sparc (Osteonectin), Tmsb4x (Thymosin beta-4), P2ry12 (Purinergic P2Y receptor 12), C1qa (Complement C1q subunit A), C1qb (Complement C1q subunit B) as features of homeostatic microglia. Activation and transition to stage 1 DAM involved downregulation of identified homeostatic genes Cx3cr1 (Fractalkine), P2ry12 (Purinergic 2Y receptor 12) and TMEM119 (Transmembrane Protein 119), along with P2ry13 (Purinergic P2Y receptor 13), Tgfbr1 (Transforming growth factor receptor beta 1), Txnip (Thioredoxin-interacting protein) and Glu1 (Glucoamylase 1), and upregulation of Tyrobp (TYRO protein tyrosine kinase binding protein - DAP12), Ctsb (Cathepsin B), Cstb (Cytstatin B), Ctsd (Cathepsin D) Apoe (Apolipoprotein E), B2m (Beta-2 microglobulin), Fth1 (Ferritin heavy chain-1), Timp2 (Tissue inhibitor of metalloprotease-2), H2-D1 (H2 class 1 histocompatability antigen) and Lyz2 (Lysozyme C-2). The expression of homeostatic genes C1qc (Complement C1q subunit C), C1qb, C1qa, Ctss, Hexb, Olfml3 (Olfactomedin-like 3), Csf1r and

Table 1. List of key genes described associated with disease-associated microglia

Gene	Gene	Gene
Homeostatic Microglia	**Stage 1 DAM**	**Stage 2 DAM**
[a]*Hexb*	[b]*CD33*	[c]*Trem2*
[a]*Cst3*	[b]*Cx3cr1*	[c]*Ankh*
[a]*Cx3cr1*	[b]*P2ry12*	[c]*Cd63*
Ctsd	[b]*P2ry13*	[c]*Cd9*
Csf1r	[b]*Tgfbr1*	[c]*Serpine2*
Ctss	[b]*Txnip*	[c]*Ctsz*
Sparc	[b]*Glu1*	[c]*Cd68*
Tmsb4x	[b]*Tmem119*	[c]*Cadm1*
Tmem119	[c]*Tyrobp*	[c]*Spp1*
P2ry12	[c]*Ctsb*	[c]*Cd52*
C1qa	[c]*Cstb*	[c]*Ctsa*
C1qb	[c]*Ctsd*	[c]*Clec7a*
	[c]*Apoe*	[c]*Axl*
	[c]*B2m*	[c]*Cts1*
	[c]*Fth1*	[c]*Lpl*
	[c]*Timp2*	[c]*Ccl6*
	[c]*H2-d1*	[c]*Csf1*
	[c]*Lyz2*	[c]*Hif1a*
		[c]*Cusb*
		[c]*Itgax*

See text for gene identification. [a]Core homeostatic Genes; [b]Down regulated genes; [c]Upregulated genes. DAM: disease-associated microglia

Cst3 remained unchanged between the different classes of microglia. Transition to stage 2 DAM involved upregulation of Trem2, Ankh (Progressive ankylosis protein), Cd9 (Tetraspanin), Cd63 (Tetraspanin-30), Serpine2 (Serine Protease inhibitor-2), Ctsz, Cd68 (macrosialin), Cadm1 (Cell-adhesion molecule-1), Spp1 (Secreted phosphoprotein-1), Cd52 (CD52), Ctsa, Clec7a (C-type lectin domain family 7 member A), Axl (AXL receptor tyrosine kinase), Ctsl, Lpl (Lipoprotein lipase), Ccl6 (C-C chemokine 6), Csf1 (Colony stimulating factor-1), Hif1a (Hypoxia inducible factor-1 alpha), Cusb (Cation efflux system protein CusB), Itgax (Integrin Subunit Alpha X) and Cst7 and downregulation of Cx3cr1, P2ry12, CD33 and TMEM119[32,33]. This study showed that Trem2 expression levels increased progressively upon activation from homeostatic to stage 1 and stage 2 DAM. This study chose Lpl (lipoprotein lipase), Timp2 (Tissue inhibitor of metalloprotease-2) and Itgax (CD11c) for antibody validation in human AD brain sections and showed strong expression in AD plaque-associated microglia. Lipoprotein lipase functions as a homodimer, and has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake. Its expression is not specific for microglia/macrophage cells. Other stage 2 DAM markers that have been studied in human AD brains include TREM2 (see separate section below), CD68 and ITGAX (CD11c). There has been controversy about the significance of CD68 expression by microglia. This is a monocyte specific lysosomal-associated membrane protein that becomes upregulated with increased phagocytosis[34]. It has been considered an activation marker, but this does raise the question whether phagocytosis markers are genuine proinflammatory markers or whether increased phagocytosis is a reparative response. CD11c (a complement C3b integrin receptor CR4) is a cell adhesion and phagocytosis marker for dendritic cells. An earlier study had shown that CD11c was constitutively expressed by microglia in brain with some upregulation in reactive microglia in AD brains[35,36]. It has been considered an activation marker, but this does raise the Table 1. List of key genes associated with Disease-Associated Microglia.

The identification of ApoE as a microglial activation marker does not concur with previous immunohistochemistry results. Possession of the APOE allele e4 is the most significant risk factor for developing sporadic AD[37,38]. Subjects homozygous for APOE e4 have up to a 7-fold greater risk of

developing AD but the mechanism(s) are still unclear. The possession of APOE e4 allele has also been associated with increased inflammation in the brain but immunohistochemistry studies have identified ApoE protein in neurofibrillary tangles, amyloid plaques and reactive astrocytes[39-42], not in microglia[43]. This is surprising as human microglia in culture and isolated brain microglia express high levels of APOE mRNA and protein. It is possible that the protein is rapidly secreted by microglia after synthesis, but evidence to date does not support ApoE as a marker for describing microglial activation states in human brain tissue. Complement C1q protein has also been studied in relation to AD pathogenesis. Antibodies to C1q have reactivity with amyloid plaques and neurofibrillary tangles in human brains[44,45]. Similar to ApoE, cultured brain-isolated microglia express high levels of complement C1q protein[46], but the reason microglia do not show immunoreactivity to ApoE or C1q in brain sections is unclear.

Core transcriptional signature of human microglia

A different approach to address the question of an AD-specific microglial gene signature was carried out by analyzing 9 different datasets obtained from profiling either sorted cells or brain tissue using unbiased correction network analysis[12]. Despite the heterogeneity between the datasets, a consensus list of 249 genes was identified, and when used to compare AD *vs*. age-matched controls, 52 genes were identified. Key genes from this list were CD84 (Signaling Lymphocytic activation molecule-5), dedicator of cytokinesis 2 (DOCK2), hepatitis A virus cellular receptor 2 (HAVCR2), Fc fragment of IgG receptor IIa (FCGR2A), linker for activation of T cells family member 2 (LAT2), CD86 (B7-2), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (PIK3CG), apoptosis-associated speck-like protein containing a CARD (PYCARD), tetraspanin-26 (CD37), myosin IF (MYO1F), leukocyte immunoglobulin like receptor A2 (LILRA2), protein tyrosine phosphatase receptor type C - CD45 (PTPRC), inositol polyphosphate-5-phosphatase D (INPP5D), CD33, Toll-like receptor-5 (TLR5), SH3 domain and nuclear localization signals 1 (SAMSN1), integrin alpha M chain - CD11b (ITGAM), dedicator of cytokinesis 8 - Zir3 (DOCK8), ribosomal protein S6 kinase A1 (RPS6KA1), colony stimulator factor-3 receptor (CSF3R), SLC7A7 (Y+L amino acid transporter 1), Oxidized low-density lipoprotein receptor 1 (OLR1), chemokine-like factor (CKLF), Parkin co-regulated gene protein (PARCG), lysozyme (LYZ), lymphocyte antigen 86 (LY86), arachidonate 5-lipoxygenase activating protein (ALOX5AP), Ras and Rab interactor 3 (RIN3), regulator of G-protein signaling 18 (RGS18), colony stimulating factor 2 receptor beta common subunit (CSF2RB), Rho GTPase activating protein 15 (ARHGAP15), Rho GTPase activating protein 45 (ARHGAP45), regulator of G-protein signaling 10 (RGS10), interleukin 10 receptor subunit A (IL10RA), macrophage scavenger receptor-1 (MSR1), bridging integrator-2 (BIN2), and cytokine-like 1 (CYTL1). Most of these have not been studied in AD brain tissues. Another study addressed the issue of microglial specific genes by performing meta-analysis of a number of different datasets[47]. This study only examined rodent gene datasets. Thirteen microglia-enriched genes were identified and 14 genes were differentially expressed in monocytes/macrophages.

Meta-analyses of multiple microglial-inflammation profiling studies

Complex analyses of a number of different gene expression profiling studies of different disease animal models, human diseased tissue and sorted murine and human cells identified multiple signatures (modules) for microglia associated with neurodegeneration. In human material, there appeared to be elevated expression of genes that were not observed in animal models. The microglia cluster of genes contain those more highly expressed in microglia compared to other myeloid cells, but these are not necessarily microglia-specific. The cluster contained Rho GTPase Activating Protein-5 (Arhgap5), C-C Chemokine receptor-5 (Ccr5), Sialomucin core protein 24 (Cd164), CUB and sushi multiple domains 3 (Csmd3), Cst3, Cx3cr1, Gcnt1 (Beta-1,3-galactosyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase), Golgi membrane protein-1 (Golm1), G protein-coupled receptor 155 (Gpr155), G protein-coupled receptor 34 (Gpr34), G protein-coupled receptor 56 (Gpr56), General Transcription Factor IIH Subunit 2 (Gtf2h2), LPS responsive beige-like anchor protein (LRBA), leucine-

rich repeat-containing protein (Lrrc3), Geranyltranstransferase (FPPS), microfibril-associated glycoprotein 3 (Mfap3), P2ry12, P2ry13, Plexin domain-containing protein 1 (Plxdc1), prostate transmembrane protein, androgen induced 1 (Prmepa1), Ras-related protein Rab-39 (Rab39), Spalt-like transcription factor 1 (Sali1), Selectin-P ligand, CD162 (Selplg), Siglech (SIGLEC-H), Toll-like receptor-3 (Tlr3), and TMEM119. A panel of 134 neurodegeneration-related genes were defined (for complete list refer to supplementary data in reference[24]). It was noted that 75% of these genes were associated with plasma membrane or extracellular space proteins. It was concluded that this was due to changes in the way microglia interact with the degenerative environment. Other genes included transcription factors Bhihe40 (Clast-5), retinoid X receptor gamma (Rxrγ), Hif1a and melanocyte inducing transcription factor (MITF), and 10 lysosome-associated genes including cathepsins (Ctsb, Ctsl and Ctsz). In a number of the models analyzed, increased expression of ApoE was consistently detected, and microglial responses to Aβ were highly dependent on Trem2 signaling. The central role for TREM2 in microglial responses has been demonstrated in network analysis showing TYROBP (DAP12), the essential adaptor protein that mediates TREM2 signaling, is a central hub gene for many of the above-listed inflammatory genes[31,48].

A recent publication employing single-nucleus transcriptomics combined with proteomic validation studies comparing AD model mice and human AD materials[26] showed that there were large differences in the glial phenotypes between AD mice and human AD samples. These investigators confirmed that the "Disease Associated Microglia" signature was dependent on expression of TREM2. There was increased expression of a number of "homeostatic" genes, including P2RY12, TMEM119 and CX3CR1, which have been downregulated in AD mouse models. This study also identified an amyloid-driven oligodendrocyte signature showing disruption in myelination, possibly driven by enhanced white-matter inflammation. In addition to the previously mentioned TREM2, APOE, HLA-DRA, and Alpha-2 macroglobulin (A2M), this study identified a number of additional markers that could be characterized in AD brains. These include Suppressor of Cytokine Signaling-6 (SOCS6), ZFP36 ring finger protein like 2 (ZFP36L2), SELPLG (Selectin-P ligand, CD162), sortilin related receptor 1 (SORL1), and chitinase-3-like protein 1 (CHI3L1), which were upregulated, and SLC11A1 (natural resistance-associated macrophage protein-1), S100A8 (S100 calcium binding protein A8), HAMP (Hepcidin), FTH1, SLC2A3 (Glucose transporter-3), Interleukin-1 beta (IL1B), Interferon Induced Transmembrane Protein 2 (IFITM2), S100 Calcium Binding Protein A9 (S100A9), regulator of G-protein signaling 1 (RGS1) and SLC25A37.

Differences between old and middle-aged human microglia

Noticeable differences in gene expression profiles were identified in microglia isolated from middle aged (young-mean age 53) and old brains (aged-mean age 94)[49]. This study produced RNA sequencing profiles of aged brain microglia and compared the results with those in another published study to show that 1060 genes were significantly upregulated in aged microglia and 1174 were downregulated[50]. Many of the significantly upregulated genes included those with genetic associations to AD risk [Table 2].

Prominent in the upregulated group were Cathepsin D (CTSD), Progranulin (GRN), Lymphotoxin beta receptor (LTBR), Translocator protein (TSPO), Cytochrome B245 alpha (CYBA), CD14 (LPS receptor), C1QA, C1QC and interferon regulatory factor-7 (IRF7), while prominent in the downregulated group were CD83, FLT1 (vascular endothelial growth factor receptor-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB), interleukin-1 beta (IL1B), cycloxygenase-2 (PTGS2), CCL4 (macrophage inflammatory protein-1 beta), CCL2 (monocyte chemoattractant protein-1), CCL3 (macrophage inflammatory protein-1 alpha), Toll-like receptor-4 (TLR4), prostaglandin E receptor-1 (PTGER1), transforming growth factor beta receptor 2 (TGFBR2) and mannose receptor C-type 1-CD206 (MRC1).

A similar approach was carried out comparing the RNA sequencing profiles of aged human microglia bulk isolated from the human parietal cortex in 39 autopsy cases. Data were compared with available datasets

Table 2. Prominent microglia genes that are altered with age[50]

Upregulated	Downregulated
CTSD	*CD83*
GRN	*FLT1*
LTBR	*ILIB*
TSPO	*PTGS2*
CYBA	*CCL4*
CD14	*CCL2*
C1QA	*CCL3*
C1QC	*TLR4*
IRF7	*PTGER1*
	TGFBR2
	MRC1

Table 3. Human microglia associated genes that change with aging[51]

Cell adhesion/axonal guidance		Cell surface receptors	
Upregulated	**Downre gulated**	**Upregulated**	**Downregulated**
CAECAM1	*ADGRE5*	*CD163*	*IFNGR1*
CDH3	*CDH12*	*CLEC2B*	*IL6R*
DOCK1/5	*CDH19*	*CLEC5A*	*LPAR1*
NLGN2	*CHL1*	*CXCR4*	*LPAR5*
NRP1/2	*ICAM3*	*IGF2R*	*MRC2*
PLXNC1	*ROBO2*	*P2RX1*	*MST1R*
PCDHGA2/4-8	*SEMA3C*	*TNFRSF14*	*NTRK2*
PDHGB2-4	*SEMA7A*	*IL15*	*P2YR12*
PTK7			*CLEC17A*
ROBO4			*TLR10*
SE MA4A			*TREML4*

of human and rodent microglia[51]. The paper contained large datasets of differentially expressed microglia genes with aging but the key finding was the altered expression of genes associated with cell adhesion, cell motility and different cell surface receptors [Table 3].

The microglia genes related to cell adhesion and axonal guidance were altered in aged microglia. Upregulated genes were: carcinoembryonic antigen-related cell adhesion molecule 1-CD66a (CAECAM1), cadherin-3 (CDH3), dedicator of cytokinesis-1/-5 (DOCK1/5), neuroligin-2 (NLGN2), neuropilin-1/-2 (NRP1/2), Plexin C1 (PLXNC1), protocaderin gamma -2,-4, 8 (PCDHGA2/4/8), protocadherin beta-2, -4 (PCDHGB-2, -4), protein tyrosine kinase-7 (PTK7), roundabout guidance receptor 4 (ROBO4), and Semaphorin 4A (SEMA4A). Downregulated genes were: ADGRE5 (Adhesion G-protein-coupled receptor CD97), Cadherin-12 (CDH12), CHL1, intercellular adhesion molecule-3 (ICAM3), roundabout guidance receptor 2 (ROBO2), Semaphorin 3A (SEMA3C), and Semaphorin 7A (SEMA7A). Immune receptor-related genes were also altered in aged microglia. Upregulated genes were: CD163 (Hemoglobin scavenger receptor), C-type lectin domain family 2B (CLEC2B), C-type lectin domain family 5A (CLEC5A), Chemokine receptor type 4 (CXCR4), insulin growth factor 2 receptor (IGF2R), purinergic receptor X1 (P2RX1), tumor necrosis factor receptor superfamily14 (TNFRSF14), and interleukin-15 (IL15). Downregulated genes were: interferon gamma receptor 1 (IFNGR1), interleukin 6 receptor (IL6R), lysophosphatidic acid receptor 1 (LPAR1), lysophosphatidic acid receptor 5 (LPAR5), mannose receptor C type 2 (MRC2), macrophage stimulating 1 receptor (MST1R), neurotrophic receptor tyrosine kinase 2 (NTRK2), purinergic receptor Y12 (P2RY12), C-type lectin domain family 17A (CLEC17A), toll-like receptor 10 (TLR10) and triggering receptor expressed on myeloid cells like-4 (TREML4).

ROLE OF CLASSICAL PRO-INFLAMMATORY CYTOKINES IN AD NEUROINFLAMMATION

The early theories of the role of neuroinflammation in AD suggested the involvement of classical

proinflammatory cytokines and chemokines such as interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ) and CCL-2. Demonstration of cytokines IL-1α, IL-1β and TNF-α in AD brain microglia has been reported but these are not widely-used markers for describing microglia in tissue[52-55]. There appears to be technical difficulties in localizing these secreted cytokines in tissue, and it should be noted that these classical cytokines do not prominently feature in the microglial disease-associated gene signatures of recent studies[10,11,24,32]. In the paper of Friedman *et al.*[24], they provide supplementary data from gene expression profiles of two studies comparing control and AD samples; neither of these detected increased expression of these classical cytokines in AD samples (supplementary data file in reference[24]).

DISCREPANCIES BETWEEN GENE PROFILING RESULTS AND TISSUE STUDIES OF MICROGLIA

Apolipoprotein E and Complement C1q

Apolipoprotein E (APOE/Apoe) and the three complement C1q genes (C1QA/C1qa, C1QB/C1qb, C1QC/C1qc) have been identified as microglial markers by expression studies, but these proteins have not been identified in microglia in AD tissue sections. APOE and C1Q proteins can be detected and are associated with Aβ plaques in AD brains[42,44], with expression of C1Q protein being detectable in neurons[45,56]. Recent experimental studies with mouse models showed that the majority of C1QA proteins in mouse brain was derived from microglia[57], and that C1Q overexpression can have a neuroprotective rather than pathogenic role[58]. As the majority of microglial gene profiling studies used rodent AD models, it is possible that these discrepancies are due to species differences in gene expression of human compared to rodent cells.

TREM2

TREM2 has become the most widely studied inflammatory/microglia marker for studies linking inflammation and AD. This came about due to the identification of heterozygous single nucleotide polymorphisms (SNP)/mutations associated with increased risk of developing AD[59,60]; and studies of Nasu-Hakola disease (NHD), which is associated with homozygous mutations in TREM2 gene or TYROBP gene[61]. TYROBP gene encodes DAP12, the essential adaptor protein that mediates TREM2 signaling. Patients with NHD, also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), develop a type of dementia similar to frontotemporal dementia. This dementia appears to be directly caused by microglial dysfunction resulting from the loss of function of TREM2 signaling. The function of TREM2 has been extensively characterized as a receptor for lipids, lipoprotein[62-64], including Apoe[65], heat shock protein 60 (HSP60), and Aβ peptide[66]. TREM2 appears to be a pattern recognition type of receptor rather than being ligand sequence specific. It is still not clear whether TREM2 is recognizing Aβ in plaques, or one of the many different plaque-associated proteins or lipids that accumulate, including lipoproteins such as ApoE and ApoJ (clusterin)[67]. An interaction of TREM2 and APOE signaling pathways has been implicated in altering microglia to a more damaging phenotype[68]. However, there are still inconsistencies about whether TREM2 signaling functions in a pro-inflammatory (damaging) or anti-inflammatory (reparative) manner[32].

If one now considers the theme of this review as to whether TREM2 expression can be used to describe the phenotypes of microglia in human brain tissue, findings on this have been scarce and divergent. Studies have consistently shown that TREM2 mRNA is highly expressed in human and animal brains and in microglia, and many mechanistic studies of TREM2 associated with disease assume that all microglia express TREM2. However, few studies have successfully localized TREM2 expression to microglia in human or rodent brain tissue. An earlier study using aged Aβ plaque-developing mice showed TREM2 expression by microglia associated with plaques[69], but two studies that used hard-fixed paraffin embedded human brain tissue with antigen retrieval concluded that TREM2 was not expressed by human brain

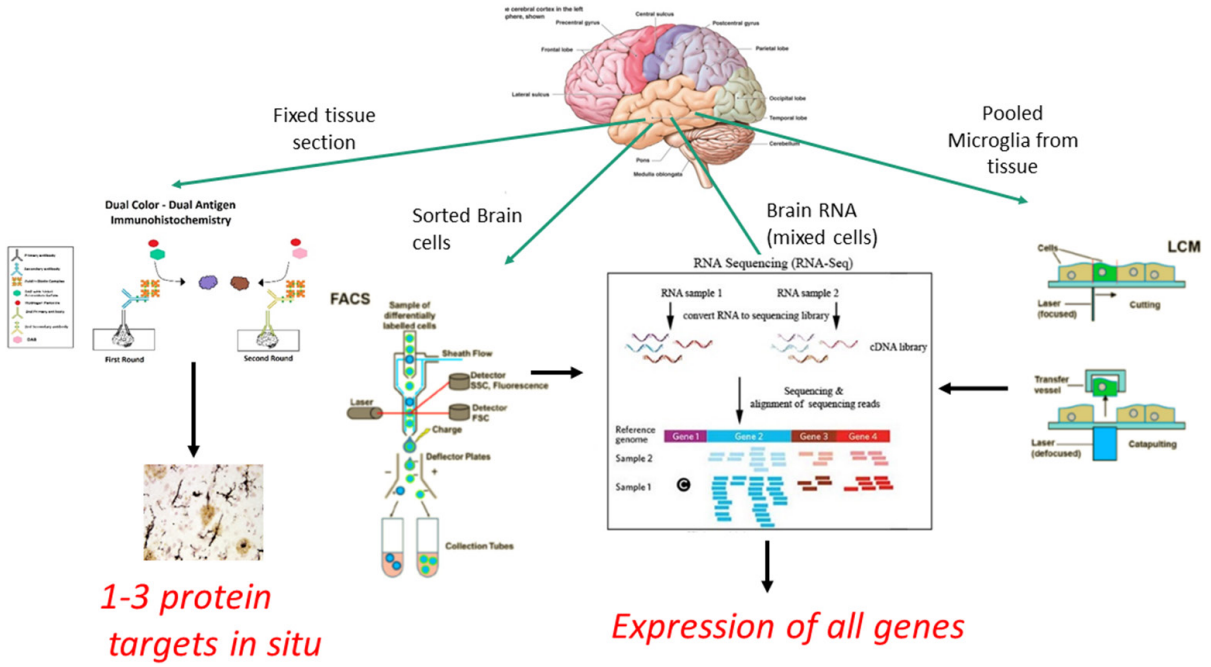

Figure 2. Technical approaches to characterizing microglia phenotypes in human brain tissue. Figure 2 illustrates different methodological approaches for defining microglia phenotypes using established immunohistochemistry methods compared to RNA gene expression profiling in sorted or single cell microglia populations, or microglia that had been laser micro-dissected from brain tissue sections

microglia[70,71]. One study that included characterization of a number of different commercial antibodies to TREM2 concluded that its expression was primarily in blood monocytes and possibly neurons[70]. Another study similarly concluded that TREM2 was expressed by monocytes or infiltrating macrophages and not by microglia[71]. These authors did not report neuronal staining[71]. The discrepancies found in these studies need to be resolved. Both of these studies carefully characterized the specificity of the TREM2 antibodies used and included positive control samples that demonstrated positive staining in blood or spleen monocytes[70,71]. By contrast, our laboratory was able to identify TREM2 immunoreactivity in different types of microglia, particularly those associated with plaques and tangles in AD brains. In our study, the expression of TREM2 by microglia was not extensive or robust, and small cells with shorter processes were identified, which is not typical of the features of tissue microglia identified with antibodies to HLA-DR or IBA-1[72].

These plaque-associated microglia might still represent infiltrating macrophages and further studies would be needed, but their localization in brain neuropil and morphology were consistent with microglia. Our study used a validated antibody, but the major difference between studies was our use of brain tissues that had not been hard-fixed or paraffin-embedded. These findings do illustrate the technical difficulties of showing TREM2-positive microglia in human brains. In further follow up studies, the demonstration of TREM2 immunolocalization using antibody AF1828 (R&D Systems, Minneapolis, MN, USA) was repeatable, but demonstration of positive TREM2 staining was not consistent, with some sections showing good microglial staining while others showing none (Figure 3: unpublished observations). Figure 3 demonstrates types of TREM2-positive microglia in an AD case (temporal cortex). In Figure 3B, the demonstration of colocalization of P2RY12 expression (brown) in TREM2 positive cells appears to define the phenotype of TREM2 expression as non-activated. P2RY12 expression has been defined to identify homeostatic microglia with this marker being downregulated with activation[28]. Our study showed increased levels of TREM2 protein in AD cases compared to control cases as measured by Western blotting[72], a finding repeated by others[73], though this latter paper did not demonstrate TREM2 immunoreactivity in microglia in tissue sections.

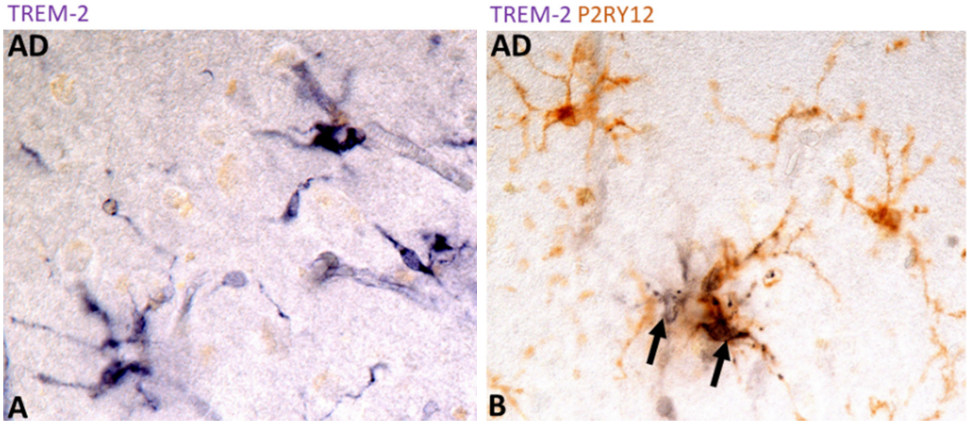

Figure 3. TREM2 expression by microglia in AD brain tissue sections. Immunohistochemical localization of TREM2 protein using antibody AF1828 (R&D systems). Sections from an AD case are shown: (A) single-stained section showing microglial-like structures immunoreactive for TREM2. Purple represents reaction with nickel-enhanced diaminobenzidine substrate; (B) double-staining (TREM2 - purple - P2RY12- brown) of AD section showing colocalization of TREM2 and P2RY12. Arrows indicate TREM2 positive microglia (purple) adjacent to P2RY12 microglia

A more recent paper suggested that there was a decrease in TREM2 protein levels in AD brains, and that neuronal and microglial TREM2 staining was also observable. These authors posited the hypothesis that brain TREM2 was derived from soluble forms of TREM2 from peripheral erythromyeloid cells and monocyte/macrophages that had trafficked into the brain[74]. These authors did not provide details on the type and fixation of the brain tissue samples employed. Replication of these findings by others would clarify the questions raised. Overall, we have demonstrated that TREM2 expression can be observed with appropriately fixed materials and a verified antibody, however, there are still inconsistencies that need to be addressed with optimal fixation methods and the availability of higher affinity antibodies. Due to the significance that experimental and gene expression studies have placed on TREM2 in AD inflammation, reliable methods to detect TREM2 *in situ* are needed to adequately define the phenotypes of TREM2-expressing microglia.

CD33

CD33, also known as Sialic acid-binding Ig-like lectin 3 (Siglec-3) is a myeloid specific cell-surface protein that is activated by binding with sialic acid-modified proteins or lipoproteins. The rs3865444 SNP resulting in a substitution of A for C in the 5' untranslated region of CD33 mRNA was found to be protective for developing AD[75]. Based on animal studies, it was concluded that high levels of microglial expression of CD33 inhibited phagocytosis of Aβ, while lower levels of CD33 present in subjects with the minor A variant of rs3865444[76] was associated with increased phagocytosis of Aβ[77]. We demonstrated by immunohistochemistry of brain sections that increased microglial expression of CD33 was evident in AD cases, particularly in plaque-associated microglia. Similar plaque-associated CD33-positive microglia were demonstrated in another study[78]. CD33 expression does not fit into the established parameters of pro- or anti-inflammatory phenotypic markers as *in vitro* activation of cultured microglia resulted in significant down-regulation of expression[76]. In addition, CD33 functions as an inhibitory receptor resulting in activation of its immunoreceptor tyrosine-based inhibitory motif (ITIM) that results in downregulation of inflammatory signaling. CD33 is not itself an Aβ phagocytic receptor, but activation could inhibit functioning of other known Aβ binding/phagocytic receptors. It has been suggested that increased expression of CD33 could be pathogenic in AD, but there is evidence that the opposite should be considered. Studies of CD33 expression in macrophages from diabetics, where expression is downregulated, showed lower CD33 expression correlated with increased levels of proinflammatory cytokine TNFα[79].

Increased levels of CD33 in AD brains might function to restrict inflammatory activation as well as inhibiting phagocytic receptors, but blocking CD33 activation could result in enhanced neuroinflammation. A clear demonstration of the interaction of CD33 and TREM2 signaling occurred in experimental mouse models[80]. The loss of CD33 in these models resulted in increased levels of key proinflammatory cytokines. Significant information on whether these proteins interact in AD brains could be obtained if high-resolution immunohistochemistry could show microglia staining for both proteins *in vivo*. The phenotypes of CD33 or TREM2 positive microglia have not been rigorously investigated to determine if they primarily express pro-inflammatory or reparative/homeostatic markers.

Progranulin

Increased expression of progranulin by microglia has also been observed in AD brains[81-83]. This protein has multiple functions including neurotrophic, anti-inflammatory and lysosomal function regulation[84]. Progranulin expression is not restricted to myeloid cells with abundant neuronal expression having been characterized. Mutations in GRN (progranulin gene) can cause some forms of frontotemporal dementia (FTD), a neurodegenerative disease associated with neurodegeneration in the frontal and temporal cortex. The mechanism for this degeneration has been associated with enhanced microglial inflammation caused by partial loss of progranulin protein and its associated activity[85]. This protein appears to be present in most brain microglia, colocalizing with lysosomal proteins in brain sections, with increased levels in plaque-associated microglia[83]. As increased levels of progranulin can be protective, upregulated expression in AD would be suggestive of a reparative stress-associated response to neurodegenerative changes. As a marker to define microglial phenotypes, progranulin has limited utility, but its continued expression by different types of microglia would suggest it is having an anti-inflammatory effect in AD affected tissue. A recent gene profiling study of middle aged compared to old brain-derived microglia showed that GRN mRNA expression was significantly higher in older microglia[49].

Toll-like receptors

Toll-like receptors (TLR) are a class of ten pattern recognition receptors associated with identifying ligands from bacteria, viruses and fungi. However, they have also been identified to have a large range of cellular ligands. Due to their demonstrated interactions with aggregated Aβ and α-synuclein, TLR2 and TLR4 have been implicated in AD and PD, though immunohistochemistry for TLR4 has demonstrated neuronal, not microglial, localization[86-88]. Activation of TLR9 by its ligand unmethylated double-stranded DNA caused increased microglial phagocytosis of Aβ in experimental AD models[89]; however, there have been no demonstrations of its localization in microglia in human brains. In a recent study focused on TLR3, native ligand double-stranded RNA and the neuronal protein stathmin[90], we demonstrated distinct microglial expression of TLR3 in human brains with increased expression in plaque-associated microglia, and in endothelial cells, but not neurons or astrocytes[91]. These results were different from previous studies and dependent on the antibody used for immunohistochemistry. TLR3 had previously been defined as a specific marker for dendritic myeloid cells including microglia[92].

Colony stimulating factor-1 receptor

The survival and proliferation of microglia is primarily dependent on the action of colony stimulating factor-1 (CSF1) and IL34, ligands for CSF1R[93]. Binding of these growth factors to CSF1R results in microglial proliferation. These growth factors have distinct structures and though their expression is regulated differently, they have overlapping properties. CSF1R expression in the brain is mainly restricted to microglia. Studies using different CSF1R antagonist administered to mice resulted in knockout of microglia from tissue with a variety of mostly therapeutic effects, although it can also be detrimental in some circumstances depending on the disease model[94-99]. These studies have defined the significance of CSF1R to microglial function. There has only been a single definitive study demonstrating localization of CSF1R in microglia in human brains[100]. This study showed that all microglia constitutively expressed CSF1R

and this was increased in pathology-associated microglia in AD brains[100]. This original finding of CSF1R expression by microglia is now 25 years old and requires replication using modern microscopic techniques to define the phenotypes of expressing microglia. We recently demonstrated increased expression of CSF1R and CSF1 mRNA in AD brains that correlated with severity of pathology, but reduced expression of IL34 mRNA in these samples[101]. One study has shown that CSF1R can be expressed in neurons in lesioned rat brains, but this finding has not been replicated[102]. Further characterization of CSF1R in AD tissue would be helpful to map the sites of microglial proliferation in tissues. These studies could be combined with markers and transcription factors associated with microglial proliferation (e.g., PU.1, Ki67).

CD14-Lipopolysaccharide receptor

A number of blood macrophage markers that have been defined are expressed at increased levels in activated cells (reviewed[103]). These defining studies have generally utilized strong activation agents such as bacterial proinflammogens including lipopolysaccharide (LPS) and IFN-γ that induce strong inflammatory responses for killing invading microorganisms. One of the activation receptors is CD14, along with Toll-like receptor (TLR)-4, one of the components of the LPS receptor[104]. CD14 has been considered a constitutive marker for macrophages and microglia, but it is noticeable that its expression in human AD brains has only been described in a single study[105]. This study identified increased CD14 expression of plaque-associated microglia. In Figure 4, we show examples of CD14 staining of microglia, but only in AD cases. Studies characterizing the expression of CD14 in microglia freshly isolated from human brains showed low levels of constitutive expression that increased significantly when isolated microglia were cultured for 2-4 days *in vitro*[106,107]. This culture-associated increase in CD14 mRNA expression was not observed for TLR4, but culture-associated decreases of a number of genes (P2RY12, CX3CR1, TNFα, TGFβ HLA-DRA, CD11b, FCγR3) were detected[107]. Increased expression of CD14 was detected by immunohistochemistry in microglia and infiltrating monocytes in sections from cases with traumatic brain injury[108].

The immunohistochemistry results presented here that follow up the earlier findings[105] suggest that using CD14 as a marker to define pro-inflammatory activation phenotypes in human brain should be reassessed. As had been encountered for other markers, successful CD14 staining was dependent on the antibody used, and also the use of antigen-retrieval methods (pH 8.0, 80 °C, 30 min). Other CD14 antibodies tested did not produce this staining of microglia under the same conditions.

CD68

There has been some controversy in the literature about the status of CD68 (designated macrosialin for rodents) as an activation or functional phagocytic marker. CD68 is a myeloid cell-specific lysosomal associated membrane protein (LAMP) whose expression is increased in cells associated with elevated phagocytic and degradative activity. Under appropriate conditions, by employing antigen retrieval methods on free-floating sections, we can demonstrate CD68 positive structures in most microglia. We have recently demonstrated that the majority of progranulin-positive, P2RY12-positive and TLR3-positive microglia were CD68 positive to some extent, irrespective of whether the sections being examined were from non-demented control or AD cases[83,91,109]. Without antigen retrieval, there was a noticeable decrease in sensitivity of detection for CD68 with many microglia showing no reactivity (unpublished observations). It is clear that being able to optimize the sensitivity of detection of CD68 (and other antigens) has a significant effect on identifying the phenotypes of cells expressing this marker.

This is a good context to discuss semi-quantitative histological findings on the expression of a series of microglial markers, including CD68, carried out in a large series of cases by Boche and colleagues[34,110,111]. These investigators were able to examine the expression of microglial markers in samples from subjects who had received the Aβ vaccine as treatment to produce circulating levels of Aβ antibodies[110]. There were significantly decreased numbers of microglia positive for CD68, CD64, CD32 and MSR-1, but not IBA-1

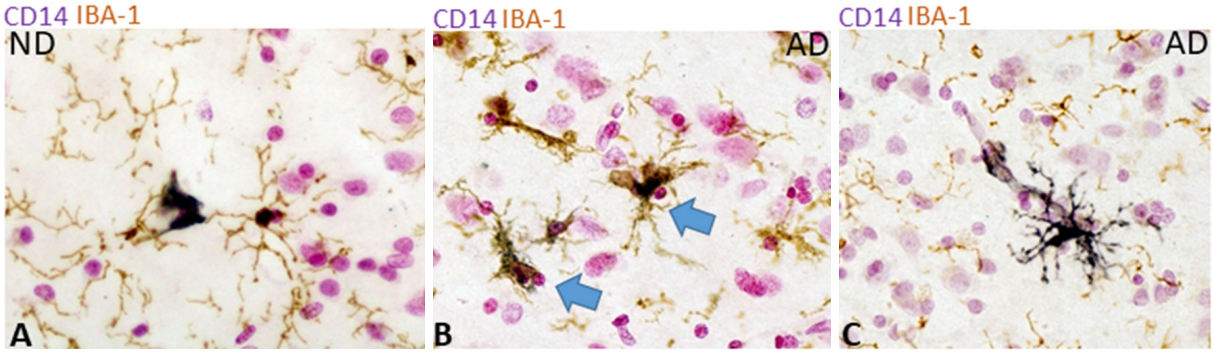

Figure 4. CD14 expression by microglia in ND and AD brain tissue sections. Immunohistochemical localization of CD14 protein using CD14 antibody (Clone 18D11, Biolegend # 812401). Sections from an ND AD case are shown: (A) Double-stained section showing strong CD14 immunoreactivity of blood monocyte (purple) but not IBA-1 positive microglia (brown). Purple represents reaction with nickel-enhanced diaminobenzidine substrate; (B, C) Double-staining (CD14 - purple - IBA-1- brown) of AD sections showing localization of CD14 and IBA-1; (B) Colocalization of CD14 and IBA-1 in microglia cells in AD case (blue arrows); (C) Strong CD14 staining of a perivascular microglia in AD section

in sections from immunized cases compared to control non-immunized subjects. The immunized cases had significantly reduced levels of Aβ[110]. In a follow-up study employing samples from 130 non-demented cases and 83 cases with AD pathology, the expression of markers CD68 (as an indicator of phagocytosis), HLA-DR, CD64, MSR1 and IBA-1 were quantified as a percentage of microglial load, and correlated with pathological and clinical indices. There were positive correlations between dementia status and microglial load for CD68, MSR-1, CD64, and a negative correlation with IBA-1 load; there was no correlation between HLA-DR load and dementia status[34]. It should be pointed out that the difference in mean load for CD68 between non-dementia and dementia with AD pathology was only 10%, confirming widespread expression of CD68 even in non-dementia brains. A similar study that focused on microglia expressing CD68, HLA-DR and IBA-1 in sections of white matter and gray matter from MS and AD cases showed stronger CD68 staining in normal white matter compared to gray matter with an increase in white matter showing MS-related demyelination[112]. Due to its intracellular location, these authors concluded that CD68 was not a good marker to describe morphological features of microglial activation.

Defining microglia by expression of homeostatic markers (TMEM119, P2RY12)

Transmembrane protein 119 (TMEM119)

TMEM119 has been identified to have a function in bone formation by promoting the differentiation of myeloblasts to osteoclasts. Its function in microglia has not been defined, but this marker was repeatedly shown to be expressed at much higher levels by microglia than macrophages, making it a good marker for specifically identifying microglia[25,28,47,113,114]. The only published report of TMEM119 expression in AD and non-demented brains demonstrated increased expression of TMEM119 mRNA in AD cases and no significant difference in the density of TMEM119 immunoreactive microglia or total protein levels in AD cases. There appeared to be an increased expression of TMEM119 in AD plaque-associated microglia[113]. This finding is contrary to the expected change in expression of TMEM119 mRNA, identified from gene expression profiling studies reported above, where microglial TMEM119/Tmem119 expression was decreased upon activation. A different result for TMEM119 was demonstrated in sections from multiple sclerosis cases with active and chronic white matter lesions. In these areas, there was a noticeable overall decrease in microglial immunoreactivity for TMEM119, but not in areas defined as pre-active white matter lesions[115].

Purinergic receptor P2RY12

P2RY12 is a receptor for adenosine triphosphate (ATP) and adenosine diphosphate (ADP). This receptor is primarily restricted to platelets and microglia. The activation of P2RY12 by ligands induces microglial

chemotaxis to sources that can include necrotic and apoptotic neurons. P2RY12 has also been identified by most microglia gene profiling studies as being a specific marker for microglia. Its expression is highest in homeostatic microglia with significant downregulation upon inflammatory activation[30]. Recent studies of P2RY12 expression by microglia in multiple sclerosis tissue sections showed downregulation in areas of active white matter lesions, similar to TMEM119, but less in areas with gray matter lesions[28,115,116]. In addition, it was observed that microglia around many Aβ plaques had reduced or no P2RY12 immunoreactivity[116]. However, our recent study further characterized P2RY12 expression by microglia in AD brains, and observed that the pathological environment (diffuse or mature plaques) had an effect on microglial P2RY12 expression[109]. Our results were noticeably different from others. We have concluded, based on these observations of increased P2RY12 expressing microglia with activation morphologies, particularly in AD brains, that this marker could be identifying other classes of microglia in addition to homeostatic/resting microglia[109]. We observed that most P2RY12 positive microglia expressed CD68 and progranulin. It was observed that highly activated appearing microglia, but also expressing P2RY12, were frequently interacting with Aβ plaques. Although our results agreed that there was an overall decrease in P2RY12 immunoreactivity, the positive cells suggest an additional function besides as a marker for homeostatic microglia.

"Alternative activation" markers (CD200 receptor, CD206, CD163)

Additional markers that have been studied for defining microglial phenotypes are CD200 receptor (CD200R), CD206 and CD163. These antigens have been defined as markers for alternative activation. CD200R was shown to be induced in cultured microglia by IL-4 and IL-13, defining it as an alternative activation marker[117,118]. CD200R has been the focus of a number of mechanistic and experimental studies because activation of this microglia/monocyte by its ligand CD200 results in downregulation of inflammatory activation signaling[119]. Activation of this receptor has significant neuroprotective effects. We showed that there was significantly decreased expression of CD200R mRNA in AD brains[117]. Despite having validated antibodies to CD200R, we could not detect CD200R protein in microglia in any brain tissue sections examined[117]. A study employing tissue from multiple sclerosis cases also could not demonstrate microglial staining of CD200R[120]. It appears then that freshly isolated microglia from human brains have very low levels of CD200R expression[120,121].

CD206 (macrophage mannose receptor C) is another widely used marker for alternative activation. Increased levels of this receptor are associated with phagocytic activity. Preliminary studies of ND and AD tissue sections with a validated antibody have failed to identify CD206-positive microglia. In our results, we can detect strong staining of vascular macrophages and perivascular macrophages [Figure 5] but not in microglia. These findings would suggest a deficit in levels of IL-4 in brain parenchyma.

However, the localization of another alternative activation marker, the phagocytic receptor CD163 has been observed in microglia in AD and PD brains, and brains affected by human immunodeficiency virus (HIV)[113,122,123]. CD163 is a high-affinity scavenger receptor for hemogloblin-haptoglobin and a low-affinity receptor for hemoglobin alone. CD163 has been widely defined as a marker of macrophages rather than microglia, particularly those that infiltrate brain tissue following stroke. It can be highly expressed by macrophages but its role in neurodegeneration is unclear. Microglia strongly expressing CD163 were shown to be plaque-associated, and CD163 immunoreactivity was present in CD68-positive microglia, suggesting a phagocytic role, and also in TMEM119-positive microglia, a homeostatic role[122]. It should be mentioned that the description of microglia based on morphology is unreliable and so, CD163 immunoreactive microglia might be infiltrating macrophages.

SUMMARY

At present, it is unresolved from studies of human brain tissues whether "activated' microglia, defined in

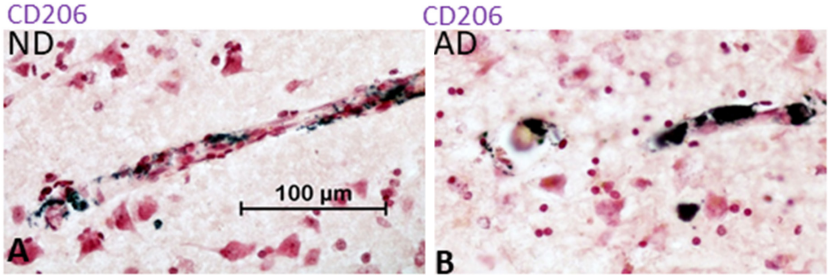

Figure 5. CD206 immunoreactivity by macrophages but not microglia in ND and AD brain tissue sections. Immunohistochemical localization of CD206 protein using antibody AF2534 (R&D systems). Sections from an ND and AD case are shown: (A) Single-stained section showing strong CD206 immunoreactivity of blood monocyte (purple). Purple represents reaction with nickel-enhanced diaminobenzidine substrate; (B) Single staining of AD section showing immunoreactivity of vascular and perivascular macrophages for CD206. No cells with morphologies of microglia were observed in sections examined. Similar findings observed by other investigators[122]

many studies based on morphology, have a predominantly pro-inflammatory phenotype or an alternative activation reparative phenotype. This remains an important issue for defining neuroinflammation in AD or other neurodegenerative diseases. Moving forward, investigators of the issues raised in this review need to consider using modern immunohistochemistry techniques that can localize multiple antigen markers to properly phenotype microglia associated with neuropathology (examples[124-126]).

CONCLUSION

Over thirty years of studies of tissue microglia in human brains and animal models of diseases have shown the increasingly complex behavior of microglial function in tissue, suggesting that classification into M1 or M2 schemes, or classical and alternative activation, is too simplistic to reflect this complexity in disease processes[127].

Recent gene expression profiling studies have shown (not unexpectedly) that there are significant differences between human and rodent microglia. This is particularly applicable when comparing microglia in diseased human brains, which have taken decades to develop a disease-phenotype, while microglia in mice brains develop disease phenotypes over weeks. Caution is thus needed in the interpretation of results from rodent models with aged humans.

Gene profiling technologies have now been applied to isolated microglia and these studies have challenged the hypothesis that there is an acute-type (microbial driven) of inflammation in human brains causing accelerated proinflammatory damage in AD. These studies have shown that many of the microglia genes expressed at increased levels reflect responses to restore homeostasis and limit inflammatory damage.

To fully understand the large amount of data from gene profiling technologies, ultimately there is the need for antibody-based studies to determine where a particular microglial marker is being expressed in the brain in relation to characteristic plaque and tangle pathology. Gene profiling studies have now identified a large number of new microglial antigenic markers that can be combined with established markers for phenotyping pathology-associated microglia.

To successfully accomplish immunohistochemistry in human brains, greater appreciation is needed for differences in the specificity and sensitivity of antibodies being used and the consequences of differences in tissue being examined (fixation, cause of death, postmortem autolysis).

To obtain consistency between laboratories in human tissue studies of microglia, some established protocols are needed to ensure that results do not simply reflect technical differences in tissue fixation and preparation, quality of antibodies being used, and sensitivity in detection of antigenic signals.

DECLARATIONS

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Authors' contributions

Conceived and wrote this article, took responsibility for the ideas presented: Walker DG

Availability of data and materials

Not applicable

Financial support and sponsorship

None.

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

The author's publications that are referenced in this review article utilized human brain tissue samples provided by the Brain Bank, BSHRI. Most of the studies referenced refer to anonymous tissue studies carried out in the USA, which are considered non-human subject research under U.S. federal regulations. Some of these studies were completed in Japan using the same tissue samples that had been transferred to Japan with the author. Tissue studies carried out in Japan were approved by the Shiga University of Medical Science Ethical Committee (Certificate No. 29-114).

Consent for publication

Not applicable.

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Neuroimmunology and Neuroinflammation

Review

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Microbiome meets microglia in neuroinflammation and neurological disorders

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Abstract

One of the emerging hot topics in biosciences is the intriguing link between gut microbial communities and its influences outside the gastrointestinal tract, such as the central nervous system (CNS), including its cognitive activities and immune responses. Beyond its neuroprotective properties, microglia are also critical for neuronal synaptic pruning and neural remodeling during CNS development. Prolonged microglia activation and neuroinflammation are considered key contributors to neurological disorders. In this regard, it is becoming increasingly important to consider the potential influences underlying the crosstalk between the intestinal microbiota ecosystem and host when determining biomarkers of disease and treatment efficacy. The commensal microbiota is critical for immune development and continuous function through the recognition of bacteria-produced and regulated metabolites. In cases of microbial dysbiosis and microglial dysfunction, chronic neuroinflammation may persist, leading to the propagation of neurological disorders. To address potential mechanisms, this review focuses on the microbiota-gut-brain axis as it relates to communication pathways that have been linked to aberrant CNS immune activity and pathology. We also address anti-inflammatory and neuroprotective mediators which may counteract these detrimental activities. Finally, we explore the potential benefits of current and novel microbiome-targeted approaches to treat neuroinflammation and consequential neurological disease.

Keywords: Microglia, neuroinflammation, neurological disorders, microbiota, gut-brain axis, vagus nerve, short chain fatty acids, hypothalamic-pituitary-adrenal axes, hypothalamic-pituitary-gonadal axes, therapeutic interventions

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INTRODUCTION

Microglia are the central nervous system (CNS) resident macrophages responsible for initiating innate immune responses to a variety of different stress and damage signals in the brain[1]. For example, when less mobile "resting" microglia recognize these signals with actively surveying processes, they become highly motile and assume an activated phenotype, facilitating the release of pro-inflammatory and cell-recruiting cytokines (e.g., IL-6, IL-12, IL-1β, and TNF-α) at the damage site[2]. After isolating and resolving the problem, it becomes critical for microglia to reestablish homeostatic conditions through the release of anti-inflammatory cytokines (e.g., IL-4, IL-10, and TGF-β) and return to the sentinel deactivated state[3]. On the other hand, prolonged microglial activation has been linked to harmful inflammatory states leading to dysfunctional brain activity and irreversible tissue damage, such as within Alzheimer's and Parkinson's diseases[4,5]. Beyond their immune functions, microglia are also important regulators of synaptic pruning and neural patterning during development and throughout adulthood[6]. Despite its evident importance in health and disease, it remains unclear whether signals to modulate microglia activity originate within the CNS only or may also occur externally from other organ/tissue systems. Identification of these additional factors related to microglial function will warrant a better understanding of causes of neuroinflammation and its relationship to CNS disorders.

Recently, one such factor has made a surprisingly strong debut within the scientific community: the vast microflora inhabiting the gastrointestinal (GI) tract has emerged as a critical player connected to multiple host systems, including those outside the GI tract. The gut microbiome (GMB) is able to modulate mucosal immunity and systemic immune activity as well as immune responses within the CNS. For example, the GMB has been demonstrated to affect the development and ongoing activity of microglia[7]. Aberrant changes to the microbiota ("dysbiosis") and dysregulated microglial activity have both been linked to some of the same neurodevelopmental, neurobehavioral and neurodegenerative disorders, including autism spectrum disorder, anxiety, depression, and Alzheimer's and Parkinson's diseases[8-12]. Of note, the GMB and its relationship to immune system maturation and developmental disorders have been extensively described in multiple reviews and are not discussed in detail within this article[13-15]. This review instead focuses on the multidirectional pathways and microbial metabolites within the microbiota-gut-brain axis as they specifically relate to neuroinflammation-induced neuropathologies. Finally, we discuss recent and novel microbiome-targeted strategies as potential treatment options for neurological disorders.

MICROBIOME, MICROGLIA AND NEUROLOGICAL DISORDERS

A mother's womb is aseptic, and, as such, we begin to develop our microbiome environments within the first few days after being born, and several factors, including method of birth, institution of breastfeeding or formula diet, and exposure to different environmental elements, determine initial microbiota compositions and continual adaptations[16]. The presence of early microbial colonization is essential for the maturation and healthy function of numerous CNS systems. The innate immune system require microbiota-induced epithelial signaling in order to correctly respond to pathogenic exposure[17]. For example, Erny *et al.*[7] demonstrated that adult germ-free (GF) mice were found to have major deficits in neuroimmune response compared to conventional controls, such as expressing reduced repertoire of cytokine and chemokine related genetic changes and inactivated microglial morphology (e.g., "failed to display rounded perikarya and small processes") in response to lipopolysaccharide (LPS) exposure. Furthermore, they were able to demonstrate that reestablishing microbiota diversity and supplementation with microbial-derived short-chain fatty acids (SCFAs), rather than total bacterial abundance load was important for partial recovery of microglial function[7]. In another example, Sudo *et al.*[18] showed that sterile-bred GF mice, devoid of microbiota, express hyperresponsive irregular hypothalamic-pituitary-adrenal (HPA) activity in response to stress compared to conventionally bred laboratory mice. The HPA axis is considered one of the main relay stations between the GMB and host CNS immune responses, which has been linked to

the development of neuropsychiatric disorders later in life[19]. Another study by Thion *et al.*[20] found that mice born from GF maternal mice expressed changes in genes regulating LPS recognition and processing *in utero* and continued to exhibit sex-specific alterations in microglial-related gene expressions postnatally. It is important to note that the timeframe when the human GMB begins to stabilize and resemble an adult composition, i.e., around three years of age, also overlaps with critical periods of CNS development, synaptic pruning, and neural remodeling[21-23]. These observations support the assertion that a complex commensal microbiota ecosystem and their metabolites are integral to the early programming of key host physiological systems.

Continuous microbiota-gut-brain axis communication may also play an important role during progression of neurological disorders occurring later in life. Clinical analyses have revealed common comorbidities and correlations between neurological symptoms and GI dysfunction, such as anxiety levels corresponding with irritable bowel syndrome symptoms[24] and GI-related symptoms associated with Parkinson's disease[25]. Furthermore, "sickness behavior" is a phenomenon descriptive of subjective changes in mood and behavior commonly found in humans and animal models of infection and illness[26]. These relationships highly suggest that GI activity, immunity and microbiome are linked to the CNS function and psychiatric disorders.

There are various methods to modulate the microbiome in order to study its direct impact on health and diseases, ranging from GF, sterile bred rodent models with overt developmental aberrations to antibiotic treatment and fecal matter transplantation (FMT)[27,28]. FMT studies have begun to reveal potential causal and therapeutic roles for the GMB through its ability to endow phenotypes from donor subjects to recipients, such as transferring anxiety-like behaviors and depressive symptoms within rodent models[29,30]. Furthermore, within the past 6 years, FMT has become a standard of care for patients suffering from recurrent *C. difficile* infections who were unresponsive to antibiotic treatments but responded favorably to the induction of healthy donor microbiota cultures[31]. Colonic samples from healthy donors were also able to improve GI and behavioral symptoms in a small ($n = 18$) cohort of children diagnosed with autism spectrum disorders[32]. Although the exact mechanisms directing microbiota-gut-brain axis influences in neurological disorders are still being investigated, communication pathways and components have been identified which are related to the systemic immune system, vagus nerve signaling, and neuroendocrine system[33].

THE MICROBIOTA-GUT-BRAIN AXIS AND IMMUNE SYSTEM

The gut microbiota, intestinal immune tolerance and homeostasis

Due to lifelong cohabitation with the intestinal microbiota, mucosal immune tolerance becomes important in differentiating between commensal and pathogenic bacteria[34]. The GMB is not hidden from immune systems but is instead active in maintaining homeostasis through "tolerogenic" signaling[35]. Toll-like receptors (TLRs) on the membrane of epithelial cells and lymphoid cells are responsible for recognizing different broad microbe-associated patterns, including bacterial membrane components, endotoxins such as LPS, and bacterial DNA[36]. TLR stimulation releases nuclear factor kappa-light-chain enhancer of activated B cells (NF-κβ) and involves activation of signaling chemokines, cytokines, and other effector proteins of humoral immune activity[37]. TLR signaling is decreased during the early weeks of development while the GMB ecosystem is being established, and immune-tolerance of bacteria is achieved when recognition of commensal bacteria-produced antigens inhibits inflammatory activation[38]. Specifically, TLR activation on the apical, microbiota-exposed membrane of epithelial cells, rather than on the basolateral membrane, inhibits the inflammatory cascade and limits immune response to microbial antigens found within the GI lumen[39]. Incorrect or incomplete immune-tolerance development can lead to autoimmune diseases, chronic inflammation, and tissue damage. The importance of the GMB in promoting immune homeostasis opens the possibility of microbiota-targeted therapeutics to reduce inflammation in response to GI diseases, such as colon cancer and colitis[40].

Microbiota-derived LPS and neural-immune interactions

The brain and connected CNS have previously been regarded as "privileged" and immunologically isolated from the rest of the body[41]. Consequently, we assumed the peripheral immune system was in place to assure healthy functioning and security for the rest of the body. Despite this separation, we have begun to identify factors outside the CNS which directly impact neurology and behaviors.

The commensal gut microbiota confers colonization protection from pathogens through nutrient and spatial niche competition, in addition to their ability to interact with the mucosal immune system and influence release of soluble IgA antibodies, antimicrobial peptides, and defensins against invaders[42,43]. However, damage to the mucosal wall, for example due to antibiotic-induced microbiota dysbiosis, overgrowth of opportunistic pathogens, and chronic inflammation, can lead to increased susceptibility to infection and permeability of the intestinal epithelial layer, known as "leaky gut", allowing luminal contents to escape into circulation and induce systemic inflammation[44,45].

The Gram-negative bacterial membrane component LPS is an endotoxin commonly utilized to study the effects of inflammation on behavior in rodent models, including voluntary ethanol intake, anxiety-like behaviors, and blood-brain barrier integrity[46-49]. Studies have demonstrated the direct effects of LPS on microglial activation and subsequent neurological pathologies and behaviors. For example, systemically introduced LPS has been shown to induce depressive-like behaviors in animals, similar to "sickness behavior" commonly comorbid with human infection diseases[50]. Biesmans *et al.*[50] showed that intraperitoneal injection of LPS increased serum levels of cytokines, including IL-1β, IL-6, TNF-α, IL-10, and MCP-1, peaking at 2 h after administration. This correlated with upregulation of CNS astrocytic immune activity biomarker glial fibrillary acidic protein, decreased locomotion, and reduced sucrose preference which indicates anhedonia associated with sickness behavior[50]. In another example, Hoogland *et al.*[51] demonstrated increased microglial activation after 48 h of LPS administration and 72 h after live *E.coli* injection. Furthermore, they found increased inflammatory cytokines within brain homogenates (TNF-α, IL-1β, MCP-1, and M-CSF) at 3 h after LPS stimulation compared to 20 h after *E. coli* infection. This indicates that *E. coli*-associated LPS endotoxins induced neuroinflammation before circulatory introduction of LPS-producing bacteria, suggesting the importance of bacterial substrates in triggering immune responses. Furthermore, blocking TLR4-LPS recognition in a rat model prevented sickness behavior following LPS challenge[52]. While unclear of its origin, whether having migrated from the gut microbiome or being derived from a brain microbiome, bacterial LPS has also been identified within the neuronal parenchyma of Alzheimer's patients[53]. Zhao *et al.*[54] observed that LPS tended to self-associate and congregate around neurons, indicated by neuronal marker NeuN- and DAPI (nuclear)-staining, within brain tissues from patients with Alzheimer's Disease compared to age-matched controls that instead expressed more punctate and dispersed LPS. Furthermore, they were able to show that primary co-cultures of human neuronal-glial cells significantly reduced DNA transcription factors when incubated with LPS[54]. Collectively, these observations suggest the critical role of the GMB and microbial endotoxins in influencing systemic and CNS inflammation related to neurological disorders.

THE GUT AND VAGUS NERVE SYSTEM INTERACTIONS

Vagal afferents and chemosensing through G-protein coupled receptors

The vagus nerve allows for bidirectional communication between the gut and brain, where afferent signaling conveys sensory information from the gut to a mesh-like system of neurons in the brain. Microglia are sensitive to intestinal microbiome changes and are effective at receiving signals from the vagus nerve to regulate neuroimmune activity and function. Gut endocrine cells (EECs) play important roles in mediating intestinal information to the CNS. They serve as chemosensors that integrate extrinsic and intrinsic signals within the gut. EECs interact with the vagus nerve by responding to different stimuli, such as nutrients, harmful toxins, and bacterial products. Through this cell-mediated sensing mechanism, EECs interact with vagus afferents by releasing serotonin, gut hormones (CCK, PYY, ghrelin, leptin,

and GLP-1) and SCFAs that target receptors located on vagus fibers. Long chain fatty acids (LCFAs) also interact with vagus receptors through cholecystokinin-dependent mechanisms.

Direct measurements of EEC activities have been challenging due to their location in the gut wall. Recently, the lab of Reimann *et al.*[55] has developed a method to directly investigate EEC activity by genetically tagging EECs with a fluorescent protein which expresses under the control of the promoter for a peptide hormone precursor proglucagon, GLP-1. Using this approach, they established the important role of G-protein coupled receptors (GPCRs) in chemosensing and their ability to activate EECs leading to the secretion of peptide hormones. GPCRs are critical for a variety of physiological functions, such as regulation of immune system, autonomic nervous system regulation, sensory (taste and smell) functions, and maintaining energy homeostasis. Recently, some GPCR chemoreceptors were found to be activated by bile acids, SCFAs, and LCFAs, which are also linked to EECs[56]. It was shown that the LCFA receptors GPR40 and GPR120 and the bile acid receptor GPR131 (TGR5) are all expressed on the surface of EECs[55]. In addition, Samuel *et al.*[57] found that isolated EECs express GPR41, a receptor for SCFAs. It has been shown that these chemosensors are located on the basolateral membrane of of EECs, which interact with the circulatory system[58]. In addition, it is likely that GPCRs co-store and co-release with gut-derived hormones, which indicate that GPCRs may be regulated by associated intestinal hormones. SCFAs and LCFAs, released from gut microbiota or derived nutritionally, can activate release of CKK hormone, which can bind to CCK-A and CCK-B receptors (CCK-r) on vagal afferents that signal the brain[59]. In response, the brain develops immune responses and triggers vagal efferent fibers to release acetylcholine (ACh), which is the principal parasympathetic neurotransmitter[60]. These observations suggest that gut dysbiosis can result in pathological changes in the levels of gut hormones and metabolites, thus influencing GPCR function and dysregulating the vagus nerve and subsequent CNS activities.

The interactions between gamma-aminobutyric acid and vagus nerve

Gut microbiota also produce a number of neurotransmitters similar to mammalian physiological systems, including dopamine, norepinephrine, serotonin, and gamma-aminobutyric acid (GABA). GABA is the major inhibitory neurotransmitter in the CNS; however, GABA receptors are expressed throughout the body, including on the vagus nerve[61]. In human intestines, GABA is produced by the microbiota populations *Lactobacillus brevis* and *Bifidobacterium dentium*. GF animals were shown to have reduced levels of GABA, suggesting that the gut microbiota is able to influence GABA levels. Furthermore, altered GABA levels have also been associated with neurological conditions, such as depression, anxiety, autism, and schizophrenia[62,63]. For example, studies into rodents were found to have reduced depressive and anxiety-like behaviors after receiving chronic administration of the probiotic *Lactobacillus rhamnosus*, which was accompanied by decreases in GABA receptor subunit mRNA expression and corticosterone levels[64]. The GABA-related reductions in behavioral effects did not occur in vagotomized rats and mice[65]. Considering this effect existed only when the vagus nerve was intact, it suggests that intestinal microorganisms regulate GABA signaling through the vagus nerve. In support of this conclusion, animal studies by Takanaga *et al.*[66] demonstrated that GABA produced by intestinal bacteria are able to cross the blood-brain barrier and influence CNS activities. In addition, the impairment of GABA-mediated neuronal inhibition associated with epilepsy might contribute to the therapeutic efficacy of vagus nerve stimulation, as was demonstrated in patients with drug-resistant partial epilepsy[67].

Vagus nerve pathways in controlling inflammation

The microbiota-gut-brain interaction through the vagus nerve plays a major role in regulating inflammation. The anti-inflammatory properties of vagus nerve function is mediated through several debated pathways, such as the cholinergic anti-inflammatory pathway, the HPA axis and the splenic-sympathetic nerve anti-inflammatory pathway.

Previous studies demonstrated that the cholinergic anti-inflammatory pathway (CAP) plays a pivotal role in controlling neuroinflammation. The CAP modulates inflammation through vagal efferent fibers that

synapse onto enteric neurons surrounding the GI tract, which can release acetylcholine[68,69]. Acetylcholine binds to α-7-nicotinic acetylcholine receptors on macrophages, including microglia, and inhibit the release of the pro-inflammatory cytokine TNF-α[70]. Other studies also illustrate the ability of the vagal nerve to regulate neuroinflammation by sensing increased peripheral pro-inflammatory cytokines[71]. As a negative feedback loop, pro-inflammatory cytokine release is prevented if increased levels of inflammation are detected through the acetylcholine-mediated anti-inflammatory signaling system[71]. Wang *et al.*[72] observed that electrical stimulation of the vagus nerve can inhibit TNF synthesis in wild-type mice but not in α-7-nicotinic acetylcholine receptor-deficient mice. Collectively, these results support the critical role of the vagus nerve in regulating microglia activity and neuroinflammation through CAP signaling.

Studies of vagus nerve stimulation have provided additional evidence for vagus nerve afferent involvement in neuroimmune modulation. For example, non-invasive vagus nerve stimulation is widely used in the treatment of drug resistant depression and has been shown to increase levels of norepinephrine[73,74]. The locus coeruleus is an aminergic brain stem nucleus which represents the main source of norepinephrine in the brain and plays a critical role as a neurotransmitter and neuroimmune modulator, including regulation of microglial activity. By activating β-receptors on the cell surface, norepinephrine affects microglia cell dynamics, which then influence neuronal activity[75,76]. These observations indicate the potential of vagus nerve stimulation to regulate microglial activity.

Recently, the inflammatory reflex was found to be located where vagus afferent fibers activate vagus efferent fibers. Borovikova *et al.*[77] reported that septic shock was prevented by vagus nerve stimulation of the distal end of the vagus nerve after injection of LPS. This effect is due to CAP activation and the binding of acetylcholine to α-7-nicotinic acetylcholine receptors in order to inhibit macrophages from releasing pro-inflammatory cytokines such as TNF-α[72]. However, the interaction between the vagus nerve and intestinal immune system is indirect because the vagus nerve does not directly interact with resident macrophages in the gut. Therefore, Rosas-Ballina *et al.*[78] suggested that the vagus nerve tends to activate the splenic sympathetic nerve through a vago-sympathetic co-activation of ventricular contractility and heart rate[78,79]. It is hypothesized that released norepinephrine from the distal end of the spleen can bind to the β2 adrenergic receptor of splenic lymphocytes. Its binding leads to the release of acetylcholine, which in turn binds to α-7-nicotinic acetylcholine receptors on splenic macrophages and inhibits the release of TNF-α[80]. However, this hypothesis is still being debated due to the controversial interaction between the spleen and the vagus nerve[81]. Furthermore, some studies demonstrate the spleen receives not only sympathetic inputs but parasympathetic inputs as well. The sympathetic inputs relay information to the spleen via the arteries while the parasympathetic inputs transfer signals at the tips of the spleen.

The vagus nerve also plays an important role within the neuroendocrine-immune axis, which can regulate coordinated neural, behavioral, and endocrine responses with the immune system in order to prevent chronic neuroinflammation. The vagus nerve recognizes peripheral pro-inflammatory cytokines released by macrophages, such as IL-1, IL-6, and TNF-α and conveys this information to the neurons within HPA pathway in order to decrease peripheral inflammation[82]. Overall, the vagus nerve has anti-inflammatory properties both through its afferent end (activation of HPA axis) and through its efferent end (activation of CAP).

THE GUT AND THE ENDOCRINE SYSTEM

In addition to the vagus nerve, intestinal microbiota are able to communicate with the CNS through hormones secreted by glands within the endocrine system. Steroid hormones take part in many critical physiological processes in our body, such as survival of stress, injury, metabolism, inflammation, salt and water balance, immune functions, and development of sexual characteristics. Studies show that gut microbiota are also able produce and regulate these hormones to affect brain activity, including the state of microglia and neuroinflammation.

Glucocorticoids through HPA axis

The HPA axis is a complex set of direct pathways and feedback interactions which include the hypothalamus, the pituitary gland, and the adrenal glands. The hypothalamus produces and releases corticotropin-releasing hormone (CRH), which can induce the pituitary to release adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal cortex, producing glucocorticoid hormones. Each of these hormones can in turn act back on the hypothalamus and pituitary in a negative feedback cycle. Glucocorticoids are corticosteroids which bind to glucocorticoid receptors present in almost every vertebrate animal cell. They can reduce certain aspects of immune activities through a feedback mechanism. Cortisol is the most important human glucocorticoid which has a variety of cardiovascular, metabolic, immunologic, and homeostatic functions. The influence of microbiota on the HPA axis depends on many factors including bacterial strain, host age and sex, and different mouse strains[83-89]. Individual strains of bacteria can regulate the HPA axis and the microbiota as a whole participate in developmentally programming stress responses[90]. Conversely, microglial activity can also affect hormone release through HPA axis. In response to cerebral insults, microglia secrete a variety of inflammatory molecules, such as cytokines, stimulating neuronal activity within the paraventricular nucleus of the hypothalamus to activate the HPA axis anti-inflammatory feedback loop to reduce prolonged neuroinflammation.

Glucocorticoids released by the HPA axis bind to glucocorticoid receptors, which are highly expressed in neurons and microglia to affect cellular responses[85,90]. Glucocorticoids work to suppress both stress and immune responses by binding to specific glucocorticoid receptors and mineralocorticoid receptors in CNS and immune cells[91]. Studies have demonstrated that acute stress induced higher levels of ACTH and corticosterone in the serum of GF mice compared to conventionally-raised control mice[85-87]. Recent targeted microarray analysis found 23 upregulated glucocorticoid receptor pathway genes in the hippocampus of GF mice compared to controls, of which six genes (*Slc22a5*, *Aqp1*, *Stat5a*, *Ampd3*, *Plekhf1*, and *Cyb561*) were confirmed by PCR validation[87]. Among these six genes, two (*Stat5a* and *Ampd3*) were upregulated in *E. coli*-derived LPS-treated mice. The GF mice demonstrated reduced anxiety-like behaviors in response to acute stress, whereas LPS-treated control mice demonstrated anti-depressive but not anti-anxiety behavior and a decrease in the basal serum cortisol levels. LPS-induced abnormal behavior was consistent with previous findings that *E. coli* colonization in GF mice enhanced the HPA axis response to stress[86]. In another study, plasma ACTH and corticosterone hormones were decreased in mice monocolonized with *Bifidobacterium infantis,* but were increased in *E. coli*-monocolonized mice. In addition, after receiving fecal samples from patients diagnosed with severe depression ("depression microbiota"), control mice exhibited anxiety- and depressive-like behaviors with parallel downregulation of *Stat5a* gene in their hippocampus compared with "healthy microbiota" recipient mice[92]. *Stat5a* is a member of STAT family encoded transcription factors, mediating signals for a broad spectrum of cytokines. The JAK2-STAT5 signaling pathway plays a critical role in mediating IL-3-induced activation of microglia[93]. Furthermore, STAT5 may play a protective role in damaged nerve cells and has been implicated in cellular functions of proliferation, differentiation, and apoptosis with relevance to processes including hematopoiesis and immunoregulation[92]. Collectively, these observations suggest that microbiota related STAT5 levels may influence neuroinflammation and related disorders.

CRH and glucocorticoids from the HPA axis have been shown to directly affect microglia activity by binding to functional CRH-R1 receptors on microglia and initiate apoptosis of microglia[94]. In that study, Ock *et al.*[94] demonstrated that CRH-induced apoptosis did not induce nitric oxide production or increase expression of pro-inflammatory genes, which indicates that CRH does not affect inflammatory activation of microglia. This mechanism has been linked to the mitochondrial pathway and induction of reactive oxygen species (ROS) production, which can damage microglia cells and promote apoptosis[95]. In support, the antioxidant *N*-acetyl cysteine inhibited CRH-induced microglial cell death suggesting that ROS was a main cause of apoptosis.

Glucocorticoid levels are strongly related to the activation of the HPA axis, and distinctively affect macrophage function. Low levels of corticosterone enhanced pro-inflammatory factors, while high corticosterone concentrations suppressed macrophage activation[96]. Steroid hormones directly target mature microglia; glucocorticoids predominantly modulate expression of glucocorticoid receptors to regulate microglial inflammatory activity[97]. Anti-inflammatory effect of glucocorticoids on microglia can reverse the pro-inflammatory function of CRH by attenuating the production of TNF-α, IL-6, and nitric oxide from LPS + IFN-γ-activated murine microglia. Physical or emotional stress may induce microglial activation in the brain as determined by changes in morphology[98,99]. The stress-induced elevation of glucocorticoids can activate microglia in rats, and chronic stress can cause a marked transition from a resting to non-resting state[100]. Temporal treatment of glucocorticoids can exhibit the opposite results[101]. Stress and administration of glucocorticoids prior to peripheral immune stimuli exerted pro-inflammatory effects on microglia, while exposure to glucocorticoids after stimuli had anti-inflammatory effects in a rodent model[101]. Corticosteroids limit microglial activation that occurs during acute stress, serving as an important endogenous suppressive signal limiting neuroinflammation[98,99]. Moreover, glucocorticoid level increases and microglial morphological complexity decreases with aging[88]. Increasing glucocorticoid levels in young mice enhanced microglial ramifications, pointing to their increased neuroprotective function. The opposite, amoeboid state of microglia renders them to move freely in the brain tissue and is indicative of inflammatory activation. Amoeboid microglia occur more frequently with aging. The effects of glucocorticoids or corticosteroids on microglia morphology are dependent on treatment time and concentration of glucocorticoids.

Estrogen through hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal axis (HPG axis) plays an important role in the reproductive and immune systems, and controls development, reproduction, and aging in animal models. The hypothalamus secretes gonadotropin-releasing hormone, the pituitary gland produces luteinizing hormone and follicle-stimulating hormone, and the gonads release estrogen and testosterone. Although the HPG axis has not been as deeply studied as the HPA axis, strong evidence suggests that estrogen has the capacity to inhibit neuroinflammatory processes and can impact immune cells, including microglial functions.

Estradiol (E2) is an estrogen steroid hormone and the major female sex hormone. Studies show that 17β-estradiol (E2) inhibits microglia activation[102] and reduces the expression of inflammatory mediators[102]. For example, E2 was able to inhibit Aβ-induced expression of scavenger receptor-A in microglia cells from an animal model of Alzheimer's disease[102]. Ovarian hormone deprivation can alter the expression of major components of estrogen and neuronal inhibitory signaling, participating in the control of microglia reactivity[103]. Moreover, aging is related to exaggerated responses to acute inflammatory stimuli, modulated by the duration of hormone deprivation. This deprivation is due to decreased estrogen receptor activity, which, despite the continuous synthesis of the receptors, induces neuroinflammation[89].

SHORT-CHAIN FATTY ACIDS IN THE GUT AND NEUROINFLAMMATION

Microbiota are able to influence brain functions through the production of metabolites such as SCFAs. In addition to being derived from dietary sources, SCFAs are also produced by the microflora in the distal small intestine and colon though the fermentation of dietary fibers. The most abundant SCFAs in the human gut are acetate, propionate, and butyrate. Acetate is used for host synthesis of lipids and cholesterol, and propionate is mostly absorbed by the liver and serves as a substrate during gluconeogenesis. Butyrate functions as the main energy source for colonic enterocytes[104]. SCFAs are mainly absorbed in both the small and large intestine through similar mechanisms, such as diffusion of the dissociated forms and through active transport by SCFA transporters[105].

High doses of systemic or locally injected butyrate has been found to exert neuroprotective effects, such as memory enhancement and cognitive function restoration[106,107]. Physiological levels of butyrate may

influence and improve neuroinflammation through different mechanisms. Butyrate is a known inhibitor of histone deacetylases (HDACs)[108,109], which control the innate inflammatory system by regulating the number of microglia cells and astrocytes[110]. Histone acetylation is a post-translational modification through epigenetic process and causes the chromatin structure to loosen by weakening electrostatic attraction between the histone proteins and DNA backbone. Activation of microglia are suppressed by this process. Therefore, increased HDACs have been shown to be involved in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases[110,111].

Butyrate is one of the most important microbial end-products of the human colon fermentation process which displays several physiological effects via different mechanisms. One function is mentioned above: butyrate is a well-established HDAC inhibitor. In addition to having a significant impact on the transcriptional system, butyrate also serves as the energy substrate. Butyrate is the primary source of energy in the colon and microbiome, which accounts for nearly 70% of ATP produced. It may appear that metabolic events in the colon are disconnected with that of the brain. However, it is impossible to ignore the immense energy demand of the brain. In this regard, energy imbalance in the brain has been noted at early stages of neurodegenerative disease such as Alzheimer's disease[112]. Another function of butyrate is its ability to activate GPCRs, as described in detail above, within the vagus nerve system section[113]. Butyrate can signal through GPR109a, which is widely expressed in colonocytes, T cells and has also been found in microglia. Butyrate is sensed by FFA2 (previously GPR43) and FFA3 (previously GPR41), which modulate the relationship between SCFAs and gut, as well as the whole body energy use[114,115].

Many studies have shown that butyrate can serve as an anti-inflammatory agent, improving gut barrier function, protecting against colon cancer and neurodegenerative diseases, such as Alzheimer's disease[116-118]. These studies demonstrate that treatment with butyrate inhibited pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6, and IL-8) and upregulated anti-inflammatory cytokines (IL-10 and TGF-β). This effect may be partly due to the inhibition of transcription factor NF-κB that controls the transcription of DNA, cytokine production, and cell survival. Aguilar *et al.*[119] demonstrated that butyrate suppressed the NF-κB signaling pathway by rescuing the redox machinery and controlling ROS, which also regulate NF-κB activation. In addition, butyrate is known to enhance and repair barrier function of intestinal epithelial cells. *In vitro* experiments have illustrated that butyrate plays an important role in the maintenance of gut-barrier integrity in order to block the translocation of LPS, which can cause immune activation[120]. For instance, butyrate leads to the upregulation of mucin 2, the most prominent mucin protein, and enhances the protection of the mucosal layer[121]. These effects of butyrate were demonstrated in Caco-2 cell cultures, which are human epithelial colorectal adenocarcinoma cells, and can form confluent monolayers *in vitro* that both structurally and functionally resemble the small intestinal epithelium. For instance, butyrate leads to the upregulation of mucin 2, the most prominent mucin protein, and enhances the protection of the mucosal layer[121].

Gut dysbiosis and reduced levels of SCFAs have been observed within neurological disease, including Pelizaeus–Merzbacher disease[122]. Unger *et al.*[123] found changes in gut microbiota and SCFAs in patients diagnosed with Parkinson's disease. Fecal SCFA concentrations were significantly reduced in Parkinson's patients compared to controls. This was associated with reduced microbiota populations of *Bacteroidetes* and *Prevotellaceae*[123]. Furthermore, some studies have demonstrated beneficial effects of SCFAs during neuronal pathologies, such as against formation of neurotoxic Aβ aggregation, which occurs during the pathogenesis of Alzheimer's disease[124]. SCFAs have been reported to increase the expression level of retinotic acid in the GI tract, which inhibits Th17 cell differentiation and promotes Treg proliferation, limiting prolonged neuroinflammation[125]. SCFAs, especially butyrate, are able to modulate immune cells and influence cell proliferation and apoptosis. For example, high concentration of butyrate induces cell apoptosis while low concentration will enhance cell proliferation[126]. Collectively, these observations support the ability of SCFAs to have a therapeutic effect on many neurodegenerative disorders.

MICROBIOME-TARGETED THERAPEUTICS ADDRESSING NEUROLOGICAL DISEASES

The conclusion of the 10-year NIH Human Microbiome Project has been integral in providing resources, methods, and discoveries linking humans and their microbiomes to health and disease[127]. The study utilized a combination of shotgun metagenomics, untargeted metabolomics, and immunoprofiling to determine host-microbiota interactions manifest in largely diverse ways, and sampling large population sizes is critical for accurately determining potential mechanisms of microbiome-linked diseases[127]. They demonstrated that microbiome composition alone was not always an accurate representation of host phenotype, and necessitated the consideration of microbial functions of the microbiota ecosystem as they interacted with host immunity, metabolism, and other interconnected activities[128]. Through this accomplishment, microbiome-targeted strategies have begun to gain interest in both studying mechanistic relationships within animal models and in the treatment of pathologies, including those related to the gut-brain axis.

Antibiotics: non-absorbable "eubiotic" rifaximin

Beyond their bacteriostatic and bactericidal effects in treating GI infections, antibiotics have been shown to negatively affect the intestinal flora, a phenomenon considered "collateral damage". Antibiotic treatment can have long-lasting negative effects on the GMB, which has been shown to decrease diversity and reduce beneficial bacteria, leading to increased susceptibility to pathogens, such as *Salmonella* and *Clostridium difficile*[129,130]. Alternatively, rifaximin, a broad-spectrum, non-absorbable antibiotic, prescribed to treat irritable bowel syndrome and traveler's diarrhea caused by *E. coli,* has shown unique qualities related to the GMB and symptoms beyond the GI tract[131]. The mechanism of rifaximin action to reduce pathogens is through binding the β-subunit of microbial RNA polymerase and inhibition of bacterial RNA synthesis[132]. However, unlike other antibiotics which commonly reduce microbiota diversity and promote dysbiosis, rifaximin exerts anti-inflammatory properties and has the "eubiotic" ability to enrich beneficial microbiota populations[133]. For example, Maccaferri *et al.*[134] found that *in vitro* treatment with rifaximin increased levels of *Bifidobacteria*, *Atopobium*, and *Faecalibacterium prausnitzii* cultured from colonic samples of patients with Crohn's disease. These changes were also accompanied by increases in SCFAs, microbial metabolites known to be important in host health, metabolism, and immune homeostasis[135]. In a rodent model of ankylosing spondylitis spinal joint inflammation, rifaximin treatment was able to inhibit TLR-4/NF-κβ signaling and decrease levels of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-17A, and IL-21[136]. Another important commensal GMB population and producer of the SCFA lactate, *Lactobacillus,* was increased in a rat model of visceral hyperalgesia with rifaximin treatment[137]. Furthermore, hepatic encephalopathy is a common complication of patients with acute or chronic liver disease that is detected through neuropsychological testing and presents as neurocognitive decline: forgetfulness, confusion, irritability, and coma at its most severe forms[138]. These symptoms are mainly a result of elevated levels of ammonia. Rifaximin was able to reduce levels of ammonia-producing intestinal bacteria without decreasing GMB diversity, while also significantly reducing hospital stay, mortality rate, and improving psychometric test performance in patients with mild and severe hepatic encephalopathy compared to other treatments[139]. These observations support alternate uses for rifaximin which may be related to beneficial changes in microbiota and SCFAs, including its indication for CNS-related disorders.

Microbial-derived metabolites: sodium butyrate

Beyond directly targeting and supplementing live bacteria in the GMB, the Human Microbiome Project stressed the importance of microbiota functions in influencing host immunity and pathologies. As a HDAC inhibitor, sodium butyrate can change the balance between two types of enzymes, histone acetylase and HDACs[140]. These two enzymes control acetylation, which is an important process in chromatin structure and gene expression associated with many diseases, such as diabetes, Alzheimer's disease, and various cancers[141-143]. Physiological doses of sodium butyrate (0.25-4.00 mM) were observed to inhibit glioblastoma cell proliferation and induce cancer cell senescence *in vitro*[143]. Pharmacological treatment of sodium

butyrate was also shown to significantly increase survival rate and delay the neuropathological sequelae in the R6/2 transgenic mouse model of Huntington's disease[106]. The findings of Arnoldussen *et al.*[144] demonstrated the beneficial effect of dietary butyrate intervention on the detrimental effects of high fat diet, including relieving high fat diet-induced cognitive impairment and dementia in humans. In addition to serving as a therapeutic agent in some specific diseases, sodium butyrate can have complementary effects when administered with other agents, such as metformin. Metformin is the most prescribed oral anti-diabetic agent, whose potential benefit in many diseases has been investigated. Recent research demonstrates that metformin is able to increase butyrate-producing populations within the gut microbiome[145,146]. Additional data indicate metformin and butyrate have anti-inflammatory effects in relation to physiological functions, including transcription, replication, and repair in the process of tumorigenesis[147]. Other SCFAs also have therapeutic effects. For example, glatiramer acetate serves as immunomodulator to reverse detrimental immune reactivity in two murine models of irritable bowel disorder. Collectively, these findings point to the therapeutic potential of sodium butyrate and other SCFAs in the treatment of various pathologies including neurological disorders.

Targeting the vagus nerve

Lewy body aggregates, constituted mainly by α-synuclein and ubiquitin, and GI dysfunctions are physiopathological characteristics of early development of Parkinson's disease[148]. Braak *et al.*[149] hypothesized that these early biomarkers initiate within the gut and then progress to the CNS via the vagus nerve and spinal cord. In support, vagus nerve-mediated brain migration of α-synuclein injected into the intestinal wall has been found in a rodent model[150]. Sander and his colleagues further indicated the correlation between the vagus nerve and cognitive fatigue in multiple sclerosis patients[151]. It is thought to be the result of the vagus nerve stimulation due to the pro-inflammatory cytokines causing changes in neural activity in brainstem and hypothalamus[152]. Furthermore, the stimulation of the vagus nerve is used in the treatment of drug resistant depression, which is the major factor for developing Alzheimer's disease. Experiments in APP/PS1 (a murine model of Alzheimer's disease) animals were performed to induce morphological changes in microglia towards a neuroprotective phenotype, which was mediated by vagus nerve activation[153]. Therefore, due to its important role in regulating the gut-brain axis through transferring microbial metabolites and neurotransmitters, such as SCFAs and GABA, manipulation of vagus nerve signaling may play a key role in modulation of some neurological conditions, including Parkinson's disease, Alzheimer's disease, and multiple sclerosis.

LIFE-STYLE INTERVENTIONS

Lifestyle interventions can affect gut microbiome composition, which influence brain activity and immune responses. Since neuroinflammation is strongly linked to neurodegenerative diseases, lifestyle alterations, such as dietary supplement and exercise, are able to play an important role in improving disease states.

Pre-/probiotic supplementation

Probiotics are living beneficial microorganisms (bacteria and yeasts), and prebiotics are the indigestible fibers which feed them[154]. Probiotics have been widely marketed and consumed as dietary supplements or as functional foods, such as "live" yogurts[154]. Probiotic treatments with *Lactobacillus acidophilus*, *L. casei*, and *L. rhamnosus* were able to affect transcription of host genes related to mucosal immunity in healthy human volunteers, supporting the ability of live bacterial cultures to affect host activities[155]. D'Mello *et al.*[26] demonstrated a probiotic mixture, VSL#3, was able to reduce "sickness behavior" by increasing novel social investigation in a liver inflammation rodent model, which was related to an increase in circulating G-CSF, reduction in TNF-α, and a decrease in activated microglia. In an *in vitro* study, peripheral blood mononuclear cells isolated from patients with Parkinson's disease were co-cultured with probiotic bacteria, *Lactobacillus* and *Bifidobacterium*, to investigate changes in innate immune cell release of inflammatory signaling markers. Probiotic strains were able to significantly reduce pro-inflammatory (TNF-α, IL-6, and IL-17A) and increase

anti-inflammatory cytokines (IL-4 and IL-10)[156]. In another study of a randomized, double-blind trial, patients diagnosed with Alzheimer's disease given a probiotic mixture for 12 weeks, exhibited a significant score improvement on the mini-mental state examination compared to controls[157].

High fiber prebiotic with or without probiotic supplementation can be a non-invasive strategy to treat neurological conditions. High fiber diets can affect gut microbiota abundance. For example, inulin is a prebiotic fiber and inulin-type fructan supplementation on the fecal microbiota is able to selectively change abundance of specific colon bacteria strains, such as *Anaerostipes*, *Bilophila*, and *Bifidobacterium*[158]. As such, high fiber supplementation has been shown to counter age-related microbiota dysbiosis[159]. Feeding mice with inulin has been shown to beneficially alter gut microbiome resulting in improved neurological outcomes through affecting gut microbiota-produced SCFAs. In support, high fiber diets, in which SCFAs can be derived, have numerous reported health benefits in reducing risk of type 2 diabetes, obesity, stroke, and cardiovascular disease. High fiber diets have been shown to increase circulating levels of butyrate, which may affect CNS function directly[160]. Collectively, these studies provide exciting evidence and demonstrate the need for further investigations into the ability of live bacteria with or without prebiotic supplementation to treat inflammation and neurological pathologies.

Diet

The microbiota composition and diversity are sensitive to host dietary habits[23,161]. Dietary factors may have pro-inflammatory or anti-inflammatory effects[162], which can indirectly affect gut microbiota by providing multiple nutrients and specific compounds. For example, data suggest that the modified Mediterranean-ketogenic diet can modulate the gut microbiome and metabolites in association with improved Alzheimer's disease biomarkers in cerebrospinal fluid[163]. The abundance of *Enterobacteriaceae*, *Akkermansia*, *Slackia*, *Christensenellaceae*, and *Erysipelotriaceae* increases while that of *Bifidobacterium* and *Lachnobacterium* reduces after modified Mediterranean-ketogenic diet treatment in subjects. A bad dietary habit, such as chronic alcohol intake, can induce neuroinflammation and neurodegeneration. Reduction of intestinal bacterial load was able to attenuate alcohol-associated CNS and gut inflammation[164]. Alcohol activated microglia and modified its cell morphology, taking on an amoeboid shape with enlarged soma and shortened peripheral processes[164].

Exercise

Exercise is considered as a protective treatment for neurodegenerative diseases[165]. Both voluntary and controlled exercise can alter the gut microbiota[166]. The microbiota composition of exercised rats was notably different from the sedentary rats with a significantly higher butyrate concentration[167]. Voluntary running has neuroprotective effects in an α-synuclein rat model of Parkinson's disease[168]. It can protect rats against neuronal loss, increase enteric glial expression, and modify gut microbiome composition in the Parkinson's disease model[169]. Exercise is also considered to enhance immune system. The vagus nerve regulates gastrointestinal inflammatory tone. Parasympathetic neuroimmune reflex depends on vagal afferent neurons for the local release of intestinal inflammatory mediators in response to pathogenic gut bacteria. For this reason, elevated vagal tone and parasympathetic influence in the resting state of athletes foster a preferential anti-inflammatory milieu through conditionally influencing microbial composition[170].

CONCLUSION AND FUTURE DIRECTIONS

Recent discoveries link the GBM and neurological disorders through the microbiota-gut-brain axis. It is also increasingly recognized that disruptions in the GBM ecosystem and its function may directly or indirectly impact CNS disease states, implicating the involvement of microglial-induced neuroinflammation and neurodegeneration. In this respect, there is bidirectional communication between the GBM and the brain, which is achieved through several pathways [Figure 1]. This communication involves the immune system, which not only supports the tolerance towards the microbiome ecosystem residing in the GI

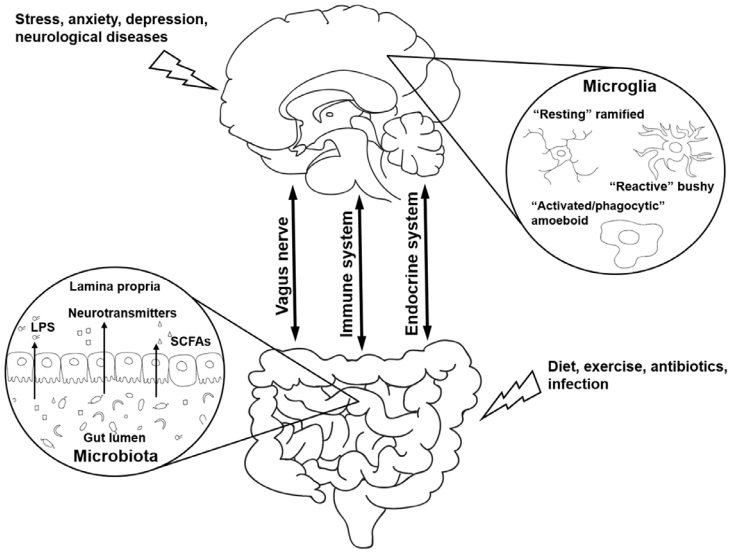

Figure 1. A schematic summarizing the pathways of gut-brain bidirectional communication with the emphasis on the immune system, the vagus nerve, and the endocrine system. The importance of the gut bacterial metabolites in this communication as well as lifestyle changes that affect microglia functioning in normal physiology and during neurological diseases with neuroinflammatory component are indicated. LPS: lipopolysaccharide; SCFAs: short-chain fatty acids

tract but also can react to dysbiosis and "leaky" gut, thus relaying this information to the CNS. On the other hand, there is the involvement of the vagus nerve in the microbiota-gut-brain interactions, which have several afferent and efferent pathways involving a variety of factors such as gut endocrine cells, neurotransmitters, and receptors. Importantly, the vagus nerve plays an important function in controlling inflammation through cholinergic and splenic-sympathetic anti-inflammatory pathways and the HPA axis. The role of hormones in the microbiota-brain bidirectional communication is also deemed important through regulation of the HPA and HPG axes. In addition, microbiota-derived metabolites, such as SCFAs and LCFAs, are integral in maintaining intestinal health and have been shown to also impact neurological health.

The GMB's critical influence on host development, immune homeostasis, and metabolism as well as involvement in the development of the CNS disorders, makes it an ideal candidate for novel preventative therapies and treatments. These strategies include the use of beneficial "eubiotic" antibiotics or other means such as lifestyle interventions (diet and exercise) aimed at reversing microbiota "dysbiosis" by targeting microbiota and their metabolites. Although in its infancy, studies into the efficacy of the microbiome-targeted manipulation and FMT to treat diseases, including those beyond the GI tract, promise interesting insights into the importance and impact of the vast and diverse microcosm residing within us every day of our lives from birth to old age and death.

Certainly, we are still in the beginning of the research trying to reveal the causative links between the GMB and brain function as it relates to neurological disorders. There is a huge untapped potential in this area of microbiome in human health and disease, which will be more appreciated with the improvement of new technologies and methods of GMB research.

DECLARATIONS

Authors' contributions

Designed the focus and scope of the review and substantially contributed to the bulk of the manuscript: Reyes REN, Asatryan L

Contributed to different sections of the review and all participated in editing and finalizing the manuscript: Reyes REN, Zhang Z, Gao L, Asatryan L

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Microglial contributions to aberrant neurogenesis and pathophysiology of epilepsy

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Abstract

Microglia are dynamic cells that constitute the brain's innate immune system. Recently, research has demonstrated microglial roles beyond immunity, which include homeostatic roles in the central nervous system. The function of microglia is an active area of study, with insights into changes in neurogenesis and synaptic pruning being discovered in both health and disease. In epilepsy, activated microglia contribute to several changes that occur during epileptogenesis. In this review, we focus on the effects of microglia on neurogenesis and synaptic pruning, and discuss the current state of anti-seizure drugs and how they affect microglia during these processes. Our understanding of the role of microglia post-seizure is still limited and may be pivotal in recognizing new therapeutic targets for seizure intervention.

Keywords: Microglia, epilepsy, neurogenesis, neuroinflammation, seizures

INTRODUCTION

Epilepsy is a neurological disorder characterized by recurrent seizures. Microglia, the innate immune cells of the central nervous system (CNS), are increasingly recognized as mediators of seizures and contributors to the epileptogenic process. The progression to epilepsy is characterized by the presence of neuroinflammation, as well as structural and molecular alterations in the brain, that subsequently lead to increased neuronal hyperexcitability and a lasting disposition towards spontaneous recurrent seizures (SRS)[1]. Microglia regulate neuroinflammation and axonal sprouting and have been reported to modulate

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neurogenesis. Following seizures, microglia are activated, functioning as resident macrophages of the brain and respond quickly to injury while trying to maintain the physiological processes under its control[2]. Changes in neuronal homeostasis are also observed, highlighting the diverse ways in which microglia could be contributing to the development of epilepsy.

This review will discuss the roles of microglia in neuroinflammation and neurogenesis, and how these contributions are altered post-seizure. We will examine microglia in the context of epileptogenesis, the process by which "the previously normal brain is functionally altered and biased towards the generation of abnormal electrical activity that subserves chronic seizures"[3]. Additionally, we will explore studies of pharmacological reagents and their effects on microglia as a therapeutic target to mitigate the epileptogenic process that drives epilepsy.

EPILEPSY

Epilepsy is a chronic brain disorder characterized by abnormal brain activity that causes seizures. The propensity to generate recurrent seizure events has neuropathological, cognitive, and social consequences[4]. Epileptic seizures are aberrant, excessive, or synchronous neuronal discharges and manifest in a variety of ways. According to the International League Against Epilepsy (ILAE), seizures are classified into three types based on their onset: generalized onset seizures do not have a determined area of origin and can affect both sides of the brain; focal onset seizures originate from one area of the brain; and unknown onset seizure when the onset is missed or obscured. Generalized onset seizures can present with a variety of manifestations that include non-motor and motor presentations: they range from absence seizures (that present with lapses in awareness, accompanied with staring into space, probably accompanied by rapid blinking and/or orofacial automatisms) to generalized tonic-clonic seizures with tonic and/or clonic spasms, and are always accompanied by loss of consciousness. Focal onset seizures may or may not be accompanied by a loss of awareness and their origin can be attributed to a specific area of the brain that causes motor or sensory changes, including taste or smell. Focal seizures may also result in a loss of awareness, manifested by a person who appears to be dazed, confused, and unable to respond to questions for several minutes. Focal seizures may become generalized if the original behavior, which was localized to one brain hemisphere, expands to behaviors that involve both sides of the brain[5]. The cause of epilepsy in many patients is not known, though acquired causes include stroke, traumatic brain injury (TBI), autoimmune disorders, infection, and tumors.

It is estimated that almost 10% of people will experience a seizure in their lifetime[6]. Epilepsy affects approximately 1.2% of the population in the United States alone[7]. Higher incidence rates have been reported in younger (early childhood and infancy) and older age groups (older than 55 years of age), while a lower prevalence is seen in the period between early adulthood and midlife[8]. The imbalance between excitatory and inhibitory neurotransmission (E/I imbalance), with a propensity towards increased excitation, is believed to be the underlying cause of seizures in epilepsy. Research demonstrates hyper-excitability during ictogenesis, when excitatory glutamatergic activity is increased while inhibitory gamma aminobutyric acid (GABA) ergic activity is dampened[9-11]. Currently, the treatment of epilepsy varies from patient to patient. Anti-seizure medications are typically the first choice of therapy for subsequent seizure prevention. When medication fails, surgery has been successful in significantly decreasing or making patients seizure free, though only a small number of patients with focal onset seizures would qualify for surgical options[12]. When surgery is not an option, patients are treated with antiepileptic drugs (AEDs). There have been > 30 medicines that have been approved by the United States Food and Drug Administration (FDA) or the European Medicines Agency (EMA). Even though many seizure medication options exist, nearly 33% of patients fail to respond to them[13]. Some patients with pharmacologically refractory epilepsy try to control seizures by exploring dietary changes, such as employing the ketogenic diet, a high fat/low carbohydrate diet which can be successful in reducing seizures in about 50% of adult

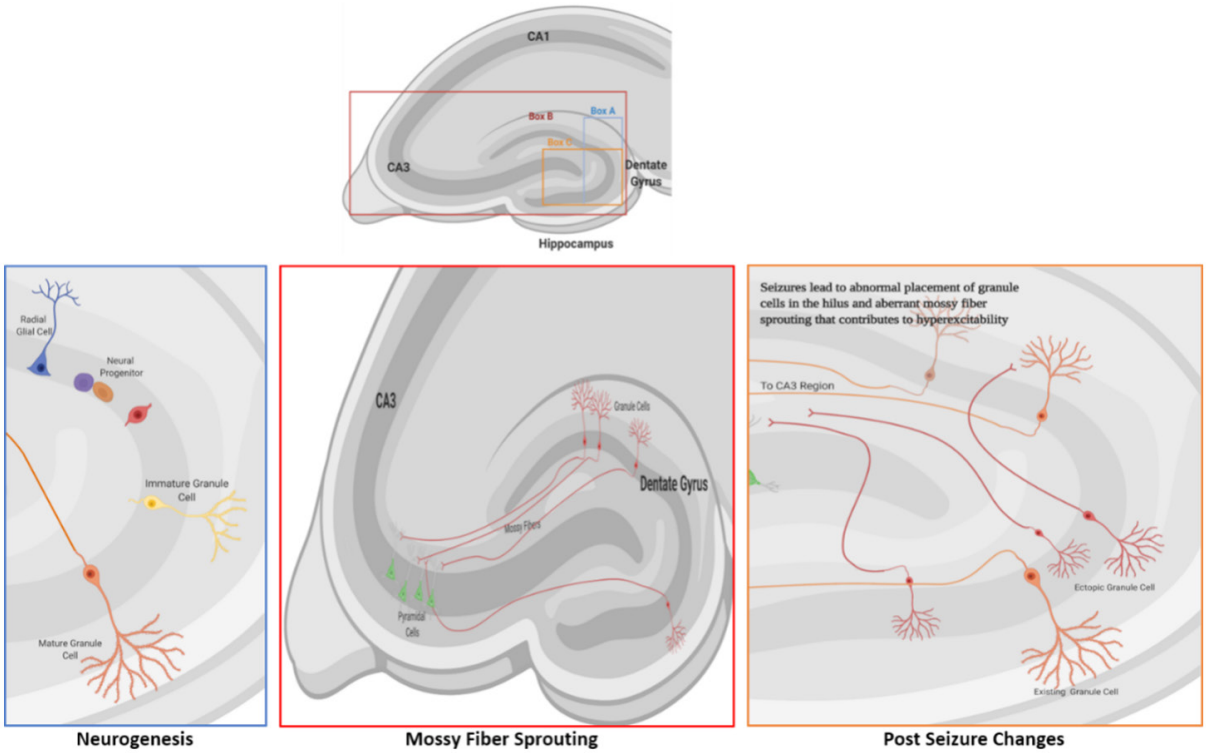

Figure 1. Granule cell neurogenesis and mossy fiber sprouting. A: Neurogenesis occurs in the dentate gyrus of the hippocampus. The cells proliferate in the subgranular zone and then migrate a short distance to the granule cell layer where they differentiate into mature granule cells; B: the axons of granule cells (mossy fibers) normally project to the cells in the CA3 region of the dentate gyrus; C: during seizures, several factors contribute to aberrant migration of granule cells that leads to their ectopic placement in the hilus. Ectopic granule cells (red cells) form functioning neural connections to the pyramidal neurons in the CA3 region and contribute to hyperexcitability and epileptogenesis through aberrant 'sprouting' along the mossy fiber pathway. Image created with BioRender.com

patients[14]. Though originally believed to result in an increase in levels of GABA production[15], there may be multiple mechanisms that contribute to its success in seizure cessation[16]. Neurostimulatory devices, such as deep brain or vagus nerve stimulation therapies, have also been used with varying success, as they help to normalize the excitatory state of the brain[17].

Epileptogenesis

Epileptogenesis is the process by which structural and molecular changes occur in the brain and predispose towards epileptic seizures[18]. The epileptogenic process can be initiated by multiple underlying causes such as tumors, infections, stroke, and brain injuries. Epileptogenesis occurs prior to an unprovoked seizure and continues beyond the event. It is a dynamic process that can occur very quickly, after brain injury or stroke, or over an extended period of time (up to months in animal models, and years in humans)[18,19]. This window presents a temporal opportunity for treatment approaches, but also provides challenges for studying the process. Understanding the pathophysiological changes that occur during epileptogenesis is a pivotal part of developing new therapies.

Changes during epileptogenesis occur in both neuronal and glial cells, all of which contribute to the dysfunction of neuronal circuits. The mechanisms underlying epileptogenesis suggest that the pathophysiological and compensatory changes are connected. Animal models of epileptogenesis have displayed histologically-detectable changes, such as sprouting along the mossy fiber pathway, neurogenesis, and gliosis [Figure 1] alterations, all of which can contribute to the potential for hyperexcitability[20]. The condition most frequently associated with mossy fiber sprouting is temporal lobe epilepsy (TLE), the

most common type of epilepsy in adults[21], but can occur in epilepsy patients without TLE[22]. Sprouting occurs when granule cell axons in the inner molecular layer (mossy fibers) project into the hilus of the dentate gyrus and CA3 region of the hippocampal formation, creating their own dendritic field. Mossy fibers synapse onto hilar mossy cells, CA3 pyramidal cells, and interneurons[23] to create de novo recurrent excitatory circuits. Aberrant sprouting in a model of TLE was reported to contribute to excitatory feedback loops of normal and ectopic granule cells[24]. Another study described aberrant mossy fibers that drive inhibitory basket cells to reduce neuronal excitability[25]. Mossy fiber sprouting is increased through the activation of several granule cell factors, such as neuromodulin and brain-derived neurotrophic factor (BDNF)[26], and involves the secretion and deposition of molecules of the extracellular matrix that facilitate aberrant growth[27-29]. The number of granule cells also affects mossy fiber sprouting. Hippocampal neurogenesis, which leads to the formation of new granule cells, is increased shortly after an epileptic seizure, but the increase is transient. The development of new granule cells, and their ectopic integration into neuronal networks contribute to aberrant mossy fiber sprouting that is evident post-seizure.

Reactive gliosis has also been identified as a contributor to epileptogenesis in genetic and chemically-induced animal models of epilepsy[30]. Activated astrocytes and microglia exhibit changes that promote network hyperexcitability[31,32]. Microglia can be activated by cytokines and monocytes circulating in blood[33], neurotransmitters released by activated or damaged neurons, or by molecules migrating across the blood brain barrier (BBB)[31]. Disruption of the BBB during status epilepticus (SE) leads to the transport of plasma proteins and immune cells into the brain. The combined effects on astrocytic functions, ion concentration changes, entry of infiltrating systemic components, and potential pathogens into the CNS may lead to neuronal dysfunction, neuroinflammation, and neurodegeneration[34]. The BBB plays a pivotal role in diseases associated with neuronal hyperexcitability such as epilepsy, TBI, and post-stroke seizure activity[35-37]. Microglia-neuron signaling had been shown initially by the release of the neuronal chemokine fractalkine, which activates the CXC-chemokine receptor 1 (CXCR1) on microglia. Neurogenesis, synaptic plasticity, and neuronal survival have all been reported to be affected by the CXCR1 signaling pathway[31]. Cytokine release of IL-1β and tumor necrosis factor-α (TNF-α) and other signals (such as HMGB1 and ATP) from activated astrocytes and microglia lead to hyperexcitability in neurons[38,39]. Precise targeting of reactive astrocytes and microglia for therapeutic intervention during epilepsy and epileptogenesis may be beneficial due to microglial involvement in the processes of neurogenesis, axonal sprouting, and neuroinflammation.

Models of epilepsy

The pursuit of AEDs has provided > 30 medications, with many that were developed in the 1980s[40]. Although several animal models of epilepsy exist, clinically validated models, ones that are validated to predict efficacy and tolerability, are limited and currently only consist of three models: the maximal electroshock (MES) seizure protocol, subcutaneous pentylenetetrazol (scPTZ) acute seizure tests, and the kindled rodent model of chronic hyperexcitability[41]. Though not validated, multiple other animal models have been developed that have contributed to the understanding of the premise of new therapeutic options[42]. Still, newer drugs continue to have similar adverse events or side effects without exhibiting greater efficacy[43]. Variation in seizure models can result in acute or chronic seizure paradigms, differences in severity, or the intervening time until seizures start[44]. Acute models lack persisting changes, like a decrease in seizure threshold or spontaneous seizures. Chronic seizure models of epilepsy accommodate a period during which epileptogenesis takes place and may better represent human epilepsy[45]. Newer models, such as the post-SE model, kindling[46], or genetic models, have become more extensively used due to their ability to result in spontaneous seizures. The kindling model, where repeated electrical stimulation leads to enhanced seizure susceptibility, is commonly utilized as it has been associated with seizure induced plasticity and provides a way to study such plasticity. Combining SRS with convulsive behavior or video-electroencephalogram (EEG) represents a more accurate epilepsy model, though it is not considered a clinically validated model for AED discovery.

The chemical induction of status epilepticus, usually by injection of kainic acid or pilocarpine[47,48], can result in animals exhibiting SRSs days to weeks after SE, and allows for the determination of post-seizure changes in the brain neuropil. Models using chemoconvulsants and kindling have provided researchers with a way to study changes in mossy fiber sprouting, neurogenesis, and neuroinflammation post-seizure.

MICROGLIA

Microglia, which make up approximately 10% of the brain's cells, are the central nervous system's primary form of immune defense. Originally thought to only serve immune response functions, they are now widely recognized to perform important functions that contribute to the development and maintenance of a healthy brain. Microglia are dynamic cells that survey their environment for injury or infection. Ramified microglia rapidly and constantly extend and retract their processes to assess the environment[49]. By evaluating their surroundings, microglia can actively participate in neurogenesis[50,51], neurotrophic functions[52], neuronal phagocytosis[53], modulation of axonal processes[54], synapse formation and pruning[55-57]. It has also been proposed that microglia aid in neurotransmitter clearance, specifically glutamate[58], due to their upregulation of glutamate transporter GLT-1 in a cortical injury model[59]. Many of these functions however, are reported to be similarly performed by astrocytes.

Microglial contribution to epileptogenesis

Models of epilepsy provide insight into neuronal and glial behavior post-seizure. Microglia sense the injury, and their activation cascade is initiated[60,61] as they migrate to the region of insult, where they then remain activated for about 4-5 weeks post-seizure[62], creating an inflammatory environment around the site of seizure onset. The extent and duration of microglial activation depends on the model used[63]. Most, though not all, chronic seizure models of epileptogenesis present a persistent inflammatory state in neural tissue[64]. After an inciting event, inflammatory cascades can either begin in the CNS, or be activated by molecules in the systemic circulation via breakdown of the BBB[65]. The seizure-induced activation of microglia can be visualized and followed non-invasively by positron emission tomography using ^{11}C-PK11195, a radiolabeled TSPO (a selective translocator protein) that is expressed at low levels in the healthy CNS, but upregulated when neuroinflammation is initiated. Although TSPO does not distinguish between microglia and infiltrating macrophages[66], its upregulation provides clear proof of the neuroinflammatory state of post-seizure CNS. Acute neuroinflammation is thought to contribute to chronic neuroinflammation states or worsen a pre-existing state[67]. Understanding how and when microglia are activated after seizures, and how they contribute over time to neuroinflammation may provide a target for downregulating or attenuating epileptogenesis.

Cytokines are signaling molecules that modulate inflammatory responses and are produced by neurons and glial cells after seizures. Interleukin-1β (IL-1β), IL-2, and IL-6 are present in the brain at low concentrations, which increase post-seizure[68]. Following seizures, mRNA expression of IL-1β, IL-6, TNF-α, TGF-β, and vascular endothelial growth factor (VEGF) were all reported to be upregulated ithe hippocampus. IL-1β may induce seizures by upregulating N-methyl-D-aspartate (NMDA) receptors on post-synaptic cells[69]. Studies also suggest that uncontrolled levels of IL-1β impair synaptic plasticity and cause neuronal dysfunction[70]. Other studies have demonstrated that IL-1β decreased GABA-mediated neurotransmission, leading to neuronal hyperexcitability and seizures[71]. When IL-1β activity was blocked, acute or recurrent seizures were reduced in rodent models[38,72,73]. Anakinra, a recombinant IL-1 receptor antagonist, was successfully used in a clinical study to treat febrile infection-related epilepsy syndrome (FIRES), demonstrating that IL-1β may be a crucial target in controlling seizure recurrence[74]. TNF-α is released by microglia and astrocytes, when low levels of glutamate are detected, to maintain neuronal excitation levels by upregulating synapses[75]. TNF-α also increases microglial glutamate release through glutaminase and gap junction regulation[76] and regulates the adhesion molecule N-cadherin, which is

involved in the organization of synapses[77]. Like IL-1β, TNF-α also affects GABA levels by increasing GABA receptor endocytosis, reducing its inhibitory action[78]. Another pro-inflammatory cytokine, IL-6, is upregulated by TNF-α and IL-1β. IL-6 has been reported to decrease hippocampal neurogenesis while increasing microgliosis, possibly contributing to epileptogenesis[79].

Changes in microglia post-seizure

The question of microglial activation status and its effects post-seizure have yet to be answered. Microglia modulate the severity of early seizures in a pilocarpine model with lipopolysaccharide (LPS) pre-conditioning[80]: ablation of microglia prior to seizure onset resulted in dramatic increases of seizure severity. Since no other cell types were affected by the method of microglia ablation[81,82], it is suggested that microglia may play a role early on in seizure induction to protect the CNS from exaggerated neuronal activity. The presence of microglia may thus be beneficial during seizure; however, evidence suggests that their activation may be detrimental post-seizure. Minocycline, a tetracyclic antibiotic that has anti-inflammatory properties, has been shown to act as an inhibitor of microglial proliferation/activation[83]. Studies that used minocycline have reported that it protects against neuronal cell death after seizures, thus indicating that microglia contribute to neurodegeneration following seizures[84]. Other studies demonstrated that a 2-week course of minocycline post-status epilepticus decreased the number, duration, and severity of spontaneous recurrent seizures, suggesting that microglia are involved in the propagation of these SRS[85-87]. It should also be noted, on the other hand, that there are studies that show only partial effectiveness by minocycline[88], or inability to reverse the increase of epileptogenesis[89,90].

Inflammatory cytokines increase neuronal excitability and are believed to contribute to epileptogenesis[91]. Though inflammatory cytokines are expressed by several cell types in the brain, microglia-specific pro-inflammatory cytokines, such as IL-1β, IL6 and TNF-α, showed increased expression three days after SE but had diminished by day 21[63]. Levels of anti-inflammatory cytokines, such as Arg1, IL-4 and IL-10, were also increased. These data contribute to the existing controversy on the role that microglia and cytokines play post-seizure. Additionally, Toll-like receptor (TLR) signaling has been implicated in the production of cytokines in seizure models. Studies have demonstrated that the downregulation of TLR3 and TLR4 activities reduces recurrent and acute seizures, respectively[92,93]. Another study showed that the activated TLR4 pathway (mediated by MyD88) was part of the molecular response contributing to a pro-inflammatory environment post-SE[94]. Matsuda *et al*.[95] reported that microglia secrete TNF-α to decrease the proliferation of neural progenitor cells (NPCs) in the subgranular zone (SGZ) and demonstrated that microglial activation is partly mediated through TLR9 post-SE. These studies emphasize the need for a better understanding of the role of cytokine signaling post-seizure.

NEUROGENESIS

Neurogenesis, the incorporation of new neurons into the hippocampus, is a controlled process that affects fundamental brain activities such as memory formation and learning. Neurogenesis, and the newborn cells generated, contribute to brain plasticity and can be followed through maturation using specific markers. The progression from newborn cells to mature neurons can be tracked using markers such as Nestin and Sox-2 for newborn cells, doublecortin and polysialylated neuronal cell adhesion molecule for immature neural progenitor cells, and NeuN for mature neurons[96]. In recent years, there has been an increased effort to determine some of the major regulators of the neurogenic process in the adult brain[97-99]. Neurogenesis, mediated by the activation and differentiation of adult neural stem cells (NSCs), has been documented to occur primarily in two regions of the adult CNS: the subventricular zone (SVZ) of the lateral ventricles, and within the SGZ of the dentate gyrus (DG) in the hippocampus[100,101]. Neurogenesis in the hippocampus will be the main focus of this section, as the hippocampal region has been intimately linked and affected by seizures and epilepsy.

In rodent models of neurogenesis, radial glia-like NSCs located in the SGZ give rise to NPCs[102]. The neurogenic process involves five intricate stages, ultimately leading to the integration of newly mature granule cells in the hippocampus. During the first stage, NSCs proliferate and generate neural progenitors in the SGZ. Stage 2 is the continuous phase of survival, where NSC and progenitor cells are lost through apoptosis, in this early part of the process. During stage 3, progenitor cells undergo fate determination and differentiate into immature neurons. In stage 4, immature neurons migrate a short distance within the granule cell layer where they continue their maturation and integrate (Stage 5) into the hippocampal circuitry, receiving input from the entorhinal cortex, and projecting axons to the CA3 (mossy fibers) and hilar regions of the hippocampus[101,103-106], which further synapse with CA1 pyramidal cells[107].

In epilepsy, while the stimuli to trigger adult neurogenesis are activated, the orchestrated differentiation process is dysregulated at various steps. The newly formed granule neurons do not integrate appropriately into the dentate gyrus, thus forming aberrant connections with other neuronal cells, and contributes to epilepsy and associated cognitive decline[108-110].

The role of microglia in physiological neurogenesis

Variations in neurogenesis properties from the embryonic stages to adulthood have been studied and show that newborn neuron populations decrease with age[111], potentially due to a lowered ability of NSCs to regenerate[112], or changes in environmental cues in the hippocampus, including an activated state of microglia[113]. Microglia have been shown to participate in neurogenesis, during multiple stages of the process through the contribution of factors that affect the proliferation and survival of NSCs[114,115]. Cognitive decline has been correlated with decreased neurogenesis[116], and studies provide support to the idea that exercise or enriched environments result in an increase in neurogenesis[117-119], which may be modulated by microglial activation[120]. A pro-inflammatory environment has been demonstrated to inhibit adult neurogenesis, while anti-inflammatory treatments were able to rescue the phenotype[121,122]. All these findings demonstrate the need to understand the role of microglia in neurogenesis that takes place in the physiological and pathological CNS. The function of microglia is most likely influenced by the environmental signals in a particular setting, which will dictate the direction of their activation status.

Microglia constantly survey their environment and are in the proximity of all cell types during neurogenesis, including newborn neurons. They are also involved in the phagocytosis of NPCs and neuroblasts in a homeostatic role for maintaining neurogenic stem cells without releasing pro-inflammatory cytokines[51]. In concordance with these data, ablating microglia in the DG inhibited adult neurogenesis by diminishing neuroblast survival[123]. Although these effects are most likely mediated by the secretion of cytokines and by microglial-regulated phagocytosis, the influence of microglia on neurogenesis also extends beyond these molecular steps and events. There is a growing body of evidence demonstrating that microglial receptors can modulate their activity in neurogenesis. For example, microglial P2Y13 receptor was recently described to contribute to microglial structural integrity. When the P2Y13 receptor is knocked out, increases in proliferation of NPCs and new neurons are observed, and this may be another way to regulate neurogenesis[124]. CX3CR1 has also been demonstrated to be involved in the regulation of adult neurogenesis: microglia have been reported to activate NPCs through CX3CR1 pathways in the hippocampus[125], and CX3CR1 null (-/-) mice exhibited impaired connectivity and aberrant synapse formation[126]. This was further supported by genetic and pharmacological inhibition of CX3CR1 signaling, which also led to aberrant neurogenesis[127,128].

Abundant data show that microglia are critical in adult neurogenesis and regulate several stages of accurate incorporation of new neurons into the hippocampal circuitry. As several seizure disorders and models manifest predominantly in the hippocampus, the effects of epileptic activity on SGZ neurogenesis is starting to be uncovered.

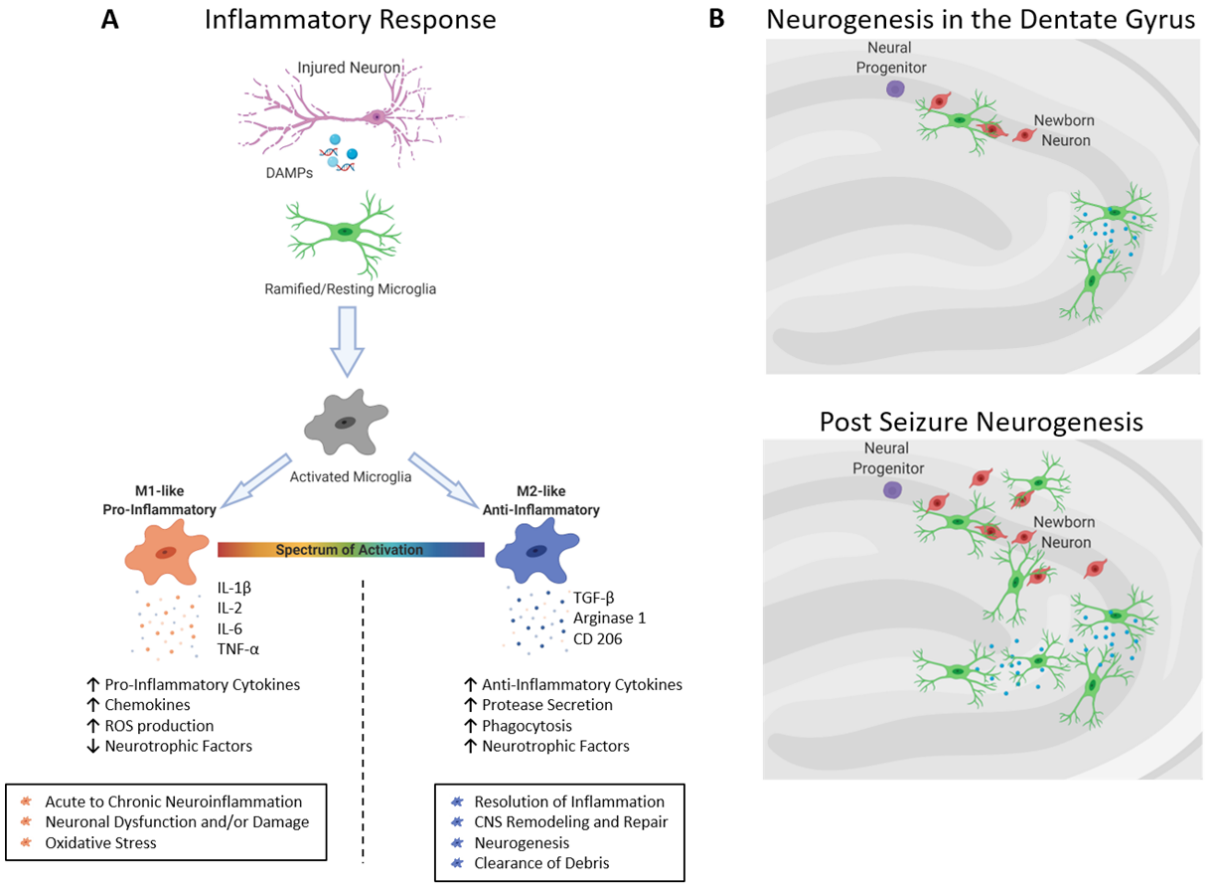

Figure 2. Microglial responses in fnflammation and neurogenesis. A: Microglia activate in response to damage associated molecular patterns (DAMPs) released by injured neurons post-seizure. Upon activation, microglial adopt one of two phenotypes: M1-like, which presents a pro-inflammatory profile that consists of decreased expression of neurotrophic factors and increased levels of pro-inflammatory chemokines and cytokines and reactive oxygen species, or M2-like, which is an anti-inflammatory response that includes the resolution of the inflammatory profile, neurogenesis and the clearance of debris; B: during neurogenesis in the hippocampus, unchallenged microglia clear cellular debris and control the number of newborn neuronal cells through phagocytosis. Post-seizure, the increased numbers of newborn cells may be cleared by microglia to reduce the potential for ectopic connections that contribute to pro-epileptic activity. Image created with Biorender.com

Neurogenesis and the pathophysiology of epilepsy

Adult neurogenesis increases following SE in animal models, resulting in an increased number of granule cells[129,130]. These additional granule cells undergo aberrant differentiation, axonal sprouting, and ectopic displacement in the hilar region of the dentate gyrus[109,131,132]. Ectopic granule cells are thought to contribute to pro-epileptic activity[133-135]; studies show that axonal sprouting and aberrant placement of granule cells were reduced when newborn granule cells were eliminated[132]. Following SE, microglia regulate the number of new granule cells through selective phagocytosis to maintain homeostasis in the dentate gyrus circuitry[136] and are capable of engulfing viable neurons in the hippocampus as well[137]. It has been suggested that microglia modulate each step (proliferation, survival, and maturation) of adult neurogenesis in both homeostasis and epileptic states[138], though their exact role in the integration of new cells has not been elucidated. Microglia may also suppress aberrant neurogenesis through the secretion of TNF-α[95], potentially leading to anti-epileptic effects [Figure 2]. Recent studies depleting microglia from the SVZ suggested that they might not be necessary for NSC proliferation[139,140], although this has not been shown in the hippocampus.

CONCLUSION

Investigation of inflammatory and neurogenic processes in epilepsy has revealed potential and critical roles of microglia in several facets of seizure generation. Epilepsy patients take AED with the aim of preventing seizures, yet studies looking at the anti-inflammatory and neurogenic effects of these drugs are sparse. Interrogating the literature for effects of AEDs in vivo on microglia, an important modulator of these processes, result in surprisingly few reports[141-143].

In vitro studies on microglial cells as mediators of inflammation have demonstrated that topiramate, a second generation AED, decreased the release of IL-1β, IL-6 and TNF-α[144]. Other AEDs such as levetiracetam, gabapentin, and phenobarbital showed slight modification in cytokine production[145]. The first generation AED valproic acid, was shown to increase IL-6 and TNF-α production in LPS-induced microglial cells[145], which contrasts with in vivo results where TNF-α and IL-1β were decreased after valproic acid treatment[143]. It was also demonstrated that the AED levetiracetam suppressed neuroinflammation and phagocytosis in a pilocarpine induced SE model[143]. Itoh *et al.*[146] reported that levetiracetam lessened microglial activation, as demonstrated by lower numbers of Iba-1 positive microglia, higher ramified shape, and low expression of pro-inflammatory cytokines. While the results of in vitro studies may eventually be applicable to the clinic, they highlight the need for clarification of the effects of AEDs on inflammation in vivo.

Studies concerning AEDs and neurogenesis are also extremely limited. Pregabalin, a widely used AED with an unknown mechanism of action, has been shown to accelerate the maturation of granule cells in the dentate gyrus[147]. In rats, lamotrigine increased the number of newborn cells in the hippocampus[148] and increased neurogenesis[149]. Valproic acid also induced neurogenesis, but these effects were not induced by phenobarbital and topiramate[149].

Epileptogenic changes in the brain are provoked by inflammation and increased neurogenic levels post-seizure. To control this process, a greater understanding of microglial contributions is needed and could provide a mechanism and target for a new generation of AEDs.

DECLARATIONS

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Authors' contributions

Co-wrote review article, edited review, generated the images: Victor TR
Co-wrote review article, edited article, correspondence: sirka SE

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Review

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Microglial heterogeneity: distinct cell types or differential functional adaptation?

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Abstract

Microglia were first characterized by del Rio Hortega about 100 years ago but our understanding of these cells has only gained traction in the last 20 years. We now recognize that microglia are involved in a plethora of activities including circuitry refinement, neuronal and glial trophic support, cell number modulation, angiogenesis and immune surveillance. Specific to immune surveillance, microglia detect threats which then drive their transformation from ramified to amoeboid cells. This morphological transition is accompanied by changes in cytokine and chemokine expression, which are far less conserved than morphology. To simplify discussion of these expression changes, nomenclature ascribed to states of macrophage activation, known as Macrophage 1 ("M1"; classic) and Macrophage 2 ("M2"; alternative), have been assigned to microglia. However, such a classification for microglia is an oversimplification that fails to accurately represent the array of cellular phenotypes. Additionally, multiple subclasses of microglia have now been described that do not belong to the "M1/M2" classification. Here, we provide a brief review outlining the prominent subclasses of microglia that have been described recently. Additionally, we present novel NanoString data demonstrating distinct microglial phenotypes from three commonly used central nervous system inflammation murine models to study microglial response and conclude with an introduction of recent RNA sequencing studies. In turn, this may not only facilitate a more appropriate naming scheme for these enigmatic cells, but more importantly, provide a framework for generating microglial expression "fingerprints" that may assist in the development of novel therapies by targeting disease-specific microglial subtypes.

Keywords: Microglia, neuroinflammation, single cell RNA-seq, NanoString

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INTRODUCTION

Microglia, the resident immune cells of the central nervous system (CNS), were first characterized 100 years ago by Pio del Rio Hortega (reviewed in[1]) but our understanding of their function remains incomplete. The best-known function of microglia is CNS surveillance whereby cell debris are scavenged during periods of pathology to maintain and re-establish a healthy homeostatic environment. However, this limited view of microglia function has evolved to include a list of other potential functions designed to establish, maintain, and when necessary, re-establish CNS homeostasis following both pathologic events and in the developing and mature healthy brain. This new appreciation for the plethora of microglial functions in both health and disease has resulted in a renewed interest in these enigmatic and mercurial cells.

At present, it is unclear how microglia are capable of mediating a wide range of activities that, in some cases, are seemingly in contrast to each other. For example, during development, microglia regulate neuronal numbers by both driving cell death[2-7] and promoting proliferation and survival[8-11]. This dichotomy of neurogenesis regulation is not limited to the developing brain since microglia both enhance[12] and deplete[13] the number of neural progenitor cells in the adult brain. Similarly, microglia regulate synapse numbers by both stripping/pruning[14-18] and stabilizing[19,20] dendritic spines and inhibitory synapses both in development and adulthood by potentially distinct mechanisms[21-24]. Furthermore, microglial regulation of cell populations is not limited to neurons as similar observations have also been reported for oligodendrocytes and astrocytes. Additionally, under pathologic conditions in the adult brain, microglia influence astrocytic phenotypes by ranging from neuroprotective to neurotoxic[25,26] and have been implicated in angiogenesis including regulation of the structure and function of the neurovasculature[27,28]. Taken together, it is becoming apparent that microglia oversee a vast array of events in the developing, healthy and diseased CNS although how such a single cell type can manage such a multitude of functions remains to be determined. Strong evidence is now emerging that microglia present as distinct subclasses but it remains to be determined if these subclasses represent intrinsically distinct cell populations, or if intrinsically similar cells are driven into functional heterogeneity dictated by changes in environmental cues provided by a highly dynamic CNS[29].

In addition to providing a brief review of several parameters and subclasses that define microglial heterogeneity, we also present novel RNA expression profile data that are consistent with the development of distinct microglial phenotypes as a consequence of distinct inflammatory environments. As presented in more detail below, we isolated cortical microglia from mice in three commonly used models to study various aspects of multiple sclerosis - cuprizone, lipopolysaccharide (LPS) and experimental autoimmune encephalomyelitis (EAE). Orally administered cuprizone results in CNS demyelination secondary to oligodendrocyte death. Intraperitoneal injection of the endotoxin LPS mediates a peripheral immune response that results in widespread CNS neuroinflammation. Similarly, EAE is induced by a peripheral injection of a bacterial exotoxin that is accompanied by Complete Freund's Adjuvant and a myelin antigen resulting in breakdown of the blood brain barrier. Although microglia from all three models presented pro-inflammatory profiles, the microglia from each expressed a unique set of factors suggesting environmental-specific responses. Although these observations are consistent with environmental cues driving heterogeneity, it remains possible, and perhaps likely, that microglia also represent intrinsically distinct populations.

Microglial heterogeneity

Currently, a prevailing thought is that microglia, which derive from the embryonic yolk sac, develop initially as a single-cell type lineage[30] and subsequently, into a heterogeneous population in the adult brain as a result of local environmental cues that define their differentiation and functional specificity[31-35]. For example, in the injured adult brain, neurons can express or secrete "find me" signals such as fractalkine/

C-X3-C motif chemokine receptor 1 (Cx3Cr1)[36] and "eat me" signals such as calreticulin/low-density lipoprotein receptor related protein[37]. These signals, which are not present in the healthy CNS, cue microglia to assume a phagocytic phenotype and to "find" and "eat" the compromised cells. Therefore, environmental cues have the capacity to drive the transformation of microglia from a surveying to a phagocytic phenotype. It remains unclear if all microglia in the neighborhood of the "find me" and "eat me" signals respond in the same manner, or if intrinsic heterogeneity results in the response from select subclasses of the microglial population.

Heterogeneity between brain regions

Some of the earliest evidence of heterogeneity within the microglial population was presented by Lawson *et al.*[38], who reported brain-region specific densities of cells with higher densities in the hippocampus and thalamus, and a lower density in the cerebellum. Although no functional differences were established, such density differences are consistent with local environmental cues regulating the microglial population. In line with this idea, De Biase *et al.*[39] reported that regional differences are tightly and specifically regulated since closely apposed nuclei within the basal ganglia present with dramatically different microglial densities[40] while other cell types in the same basal ganglia nuclei present with uniform densities, indicating that differential cell densities are not dictated by the spatial constraints of the tissue. Precisely how these region-specific differences are regulated remains to be fully explained although region-specific self-renewal rates have been presented[41] and it is possible that region-specific cues regulate proliferation and, ultimately, cell density[42]. Moreover, factors that regulate microglia numbers in the embryonic brain *vs.* the adult brain may also differ[43,44], which would be consistent with local cues defining both distribution and heterogeneity within the microglial population. This concept was supported by Grabert *et al.*[45], who demonstrated that microglia have regionally distinct transcription profiles.

Heterogeneity between sexes

Variations in cell density and transcription profiles are not limited to regional differences as similar distinctions have also been reported between microglia from male and female mice. Male mice present with more microglia in the cortex, hippocampus, dentate gyrus, and amygdala in early postnatal brains. As the mice mature, these densities flip with female mice presenting with a greater cell density in these regions[46]. Although there is no direct evidence that sex-dependent differences in cell density are responsible for functional differences, studies have shown that male and female microglia are functionally distinct and respond differently to noxious stimuli[47,48]. For example, Nelson *et al.*[49] and Yanguas-Casás *et al.*[40] showed that female microglia have a greater phagocytic capacity but male microglia have greater migratory activity under both basal and interferon γ-induced inflammatory conditions. Guneykaya *et al.*[50] then reported that male microglia display a higher antigen-presenting capacity as compared to female cells. Interestingly, microglia may also play a role in sex determination since the inhibition of microglial activity in male rodent neonates, at an age critical for sex determination, resulted in the reduction of masculine dendritic spine density and altered copulatory behavior in adults[51]. A potential caveat to this work, however, was that microglial activity was inhibited by minocycline, which is a broad spectrum antibiotic that is known to target both T cells and astrocytes[52,53].

Intrinsically defined heterogeneity?

The mechanisms responsible for these sex differences are not known and transcriptomic studies comparing male and female microglia reveal expression differences in both the healthy and perturbed states[48,50,54,55]. Whether microglia are intrinsically distinct between males and females, or if the local sex-specific environment differentially regulate male and female cells remains to be determined. Microglia from male *vs.* female mice express different sex hormone receptors[56-58] however, and present with sex-specific outcomes when exposed to these hormones[49,51,59-61]. Independent of sex, microglia have also been shown to express different levels of markers in the adult, unchallenged brain[62]. Bertolotto *et al.*[62] showed that microglia

express varying levels of keratin sulfate proteoglycan (KSPG) and these microglia are not uniformly distributed throughout the brain, with high concentrations in the hippocampus, brainstem and olfactory bulb while few were found in the cerebellum and cortex. The presence of these $KSPG^+$ microglia was independent of development though, since they were found in the same regions of both the neonatal and adult CNS. Moreover, microglia have also been shown to respond differently to the same stimuli[63,64]. Although consistent with the involvement of environmental cues in defining subclasses, these findings are also consistent with microglia being intrinsically distinct and independent of environmental influences.

Heterogenic microglial morphology

Amoeboid vs. ramified microglia

Perhaps the most recognized heterogenic aspect of microglia is their morphology. Two main classes have been identified - amoeboid-like, with few processes; and ramified, with numerous thin, highly-branched processes. Following initial colonization of the embryonic CNS, the majority of microglia present with an amoeboid-like morphology[65,66]. With CNS maturation, microglia transform their shape with brain region specificity. In the steady state CNS, amoeboid-like microglia are more abundant in perivascular white matter regions. In contrast, the extent of ramified microglia varies among regions with cerebellar microglia presenting with a less ramified morphology compared to microglia in the cortex[38,67,68]. Interestingly, Hanamsagar *et al.*[69] reported heterogeneity with regard to sex as microglia from male rodents presented with a greater and more complex process of arborization, and exhibited a greater change in process morphology following LPS perturbation as compared to their female counterparts. With age, and as the local environment changes, amoeboid-like microglia become more ramified while ramified microglia transition into amoeboid-like microglia, exhibiting greater phagocytic activity and releasing pro-inflammatory cytokines following pathologic insult[70,71]. Although the use of the amoeboid/ramified classification provides a simple approach for discussion, microglial morphologies present a spectrum of shapes and a two-class scheme is insufficient to accurately describe microglial morphologic differences.

Dark microglia

Recently, a new class of microglia was identified based on morphology. These microglia are "dark" based on their electron dense cytoplasm and are observed in non-homeostatic conditions[72]. Dark microglia exhibit signs of oxidative stress including condensed cytoplasm and nucleoplasm (consistent with their name), disrupted mitochondria and dilated endoplasmic reticulum, and are frequently observed extending processes toward synaptic clefts consistent with a role in pathologic synaptic pruning. Although their precise role remains to be fully determined, Bisht and colleagues[72] have proposed that these cells constitute a subclass of hyperactive microglia with dysregulated interactions with synapses. If correct, these cells may play a critical role in the progression of a plethora of neurodegenerative diseases with known synaptic loss[73] including Alzheimer's Disease[74,75] and multiple sclerosis[76,77].

Axon initial segment-associated microglia

Baalman *et al.*[78] have also presented evidence of a subset of microglia known as axon initial segment-associated (AXIS) microglia[78]. AXIS microglia, which comprise ~8% of cortical microglia, establish an intimate association with the neuronal cell body and the proximal axon, in contrast to "satellite" microglia that associate with the neuronal cell body and proximal dendrites instead[68]. AXIS microglia, which are initially observed at postnatal day 9 and persist through adulthood, contact both inhibitory and excitatory neurons but present with a significant preference for axon initial segments (AISs) of excitatory pyramidal neurons of layer V of the cortex[78]. The function of AXIS microglia is not known but they may provide trophic support for the neuron and the AIS.

Upon activation following a controlled cortical impact (CCI)-induced traumatic brain injury, the association between CNS microglia and the AIS is lost, consistent with the regulation of microglial

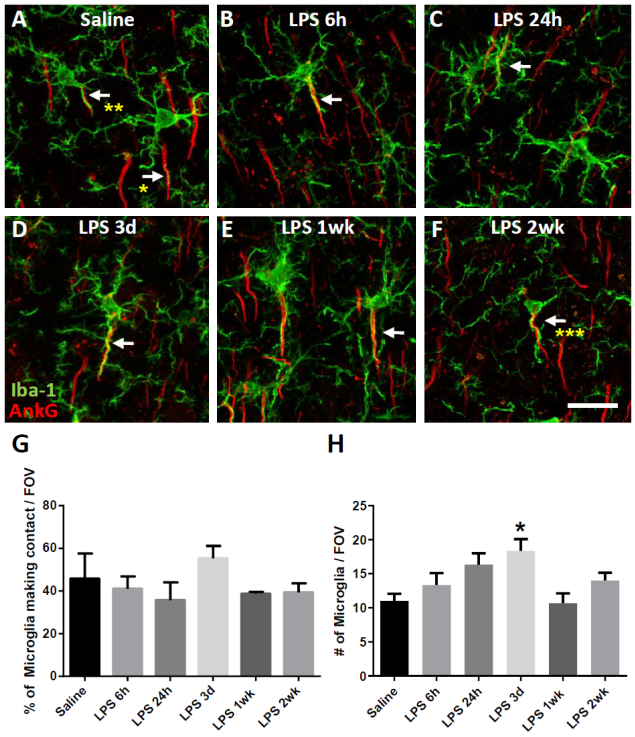


Figure 1. Frequency of microglial-AIS contact is not altered in LPS-induced neuroinflammation. Female c57black6 mice were given a single intraperitoneal injection of LPS (5 mg/kg) or vehicle (0.9% saline, 10 mL/kg). Confocal z-stacks spanning an optical thickness of 25 μm, using a pinhole of 1 Airy disc unit and Nyquist sampling (optical slice thickness, 0.48 μm), were collected from neocortical layer V for each of six sections (spanning 1.1 mm anterior to the bregma to 2.5 mm posterior to the bregma) per mouse, resulting in 12 images per animal (n = 4-6 animals per treatment group). Microglial-AIS contact was quantitatively analyzed at 6 h-2 weeks post-injection in a blind manner using Volocity™ 3D Image Analysis Software, allowing each confocal z-stack to be observed in three dimensions. The number of microglia, AISs, and contact points in each double immunolabeled z-stack was counted manually. Contact points along the six edges of the z-stacks were excluded from analysis. A-F: double immunolabeling of Iba-1 and AnkG revealed that microglia (Iba-1, green) contact AISs (AnkG, red) (white arrows) in the cortex of saline- and LPS-injected mice; G: the mean ± SEM of microglia making AIS contact per FOV in saline- and LPS-treated mice as a percent of saline controls. Quantitation of confocal z-stacks revealed that ~45% of microglia contact AISs in the cortex of saline-injected control mice. Contact was defined by co-localization of Iba-1 and AnkG and included process touching (A, yellow single asterisk), process alignment (A, yellow double asterisk), and process wrapping (F, yellow triple asterisk) as defined by 3D analysis. No change in the percent of microglia making contact was observed throughout the course of LPS-induced neuroinflammation; H: the mean ± SEM of the number of Microglia/FOV. A significant increase in the number of microglia was observed at 3 days post-LPS injection. Data were statistically compared by one-way ANOVA where mean differences were significant as assessed using Tukey's post hoc analysis. An asterisk indicates a significant difference (P < 0.05) from saline. Scale bar = 20 μm. LPS: lipopolysaccharide; FOV: field of view; AISs: axon initial segments

function and response by the local environment. Interestingly, our laboratory has also reported contact between microglia and the AIS[79,80]. Using three dimensional (3D) analysis encompassing multiple types of contact, which was defined by colocalization of ionized calcium binding adaptor molecule 1 (Iba-1) and AnkyrinG, termed (1) process touching, (2) process wrapping, or (3) process alignment [Figure 1], we found that ~45% of microglia in cortical layer V contact AISs. In contrast to the loss of contact observed following CCI injury, we observed a maintained [Figure 1], and even increased[79], association between the microglia and the AIS following inflammatory insults of LPS injection and EAE induction, respectively. The difference in AXIS microglial responses to insult is intriguing and requires further study to fully elucidate microglial response to pathology.

Herein, we have reviewed several subclasses of microglia that have been defined based on morphology; however, it is unclear if these subclasses are truly distinct, or if they are merely the consequence of artificial classifications based on techniques used for identification, and loose criteria for defining subtypes (reviewed[29]). If the latter is the case, then there is likely considerable overlap among these

subclasses. For example, we reported that under certain pathologic conditions, microglia exhibit both an increased association with, and disruption of the AIS[79]. It is possible that these microglia are no longer providing trophic support for the AIS, a suggested function of AXIS microglia by Baalman *et al.*[78] during homeostatic conditions, but are actively attacking the AIS, perhaps through the release of reactive ions[81]. If so, then could these microglia, which we characterized using immunocytochemical approaches, in fact, be "dark microglia", which are identified by electron microscopy? Studies to address this question are currently underway. In addition, are the AXIS microglia, as described by Baalman *et al.*[78], the same subclass as the microglia we have described making AIS contact? Based on work from Baalman *et al.*[78], it is likely that the AXIS microglia are supporting the neuron and the AIS; but based on our observations, the microglia may be mediating AIS disruption instead. Answering these questions is essential for accurately classifying microglia but more importantly, it would help to fully understand the role that these mercurial cells play under different conditions.

Heterogenic microglial transcriptomes

Surveying vs. reactive ("M1/M2") microglia

In an effort to more conclusively characterize microglia and to elucidate their functions, morphologic characterizations have been complemented by molecular classification studies. Initial attempts were based on presumed states of activity based on limited expression profiles. Simply, microglia were classified as either "activated" or "resting" but both terms are misleading. Microglia are never "resting" as we now recognize that they are constantly extending and retracting their processes to survey their surroundings[82,83]. As a result, the term "surveying", which more accurately represents the state of activity of microglia, even under homeostatic conditions, is now used in place of "resting". Similarly, a more appropriate term for "activated" is "reactive". "Activated" implies a lack of activity until microglia are stimulated. Microglia are constantly active however, and upon detection of changes in the environment, become "reactive".

Reactive microglia have been further divided into "M1" and "M2" states, referring to the classical (pro-inflammatory) and alternative (resolving/anti-inflammatory) phenotypes based on expression profiles. The "M1" and "M2" nomenclature is a naming scheme originally derived from the T cell literature and applied to macrophages based on their state of activation *in vitro* following exposure to either the T helper type 1 (Th1) cytokine interferon gamma (IFN-γ) for the "M1" phenotype, or the T helper type 2 (Th2) cytokine interleukin 4 (IL-4) for the "M2" phenotype[84]. Based on speculation of similar functions between macrophages and microglia, the "M1" and "M2" classification was then applied to microglia. The advantage of the "M1/M2" classification is that it provides a simplified nomenclature to distinguish between microglia in functionally distinct states. However, these distinct states are frequently identified by a small subset of surface markers, which limits resolution required for appreciating heterogeneity that is defined by the entire transcriptome. Moreover, this naming scheme is based on assumptions that cannot be confirmed under close scrutiny. At best, the "M1/M2" classification is inadequate for accurate description of the complex functions of these cells (reviewed by[85,86]). With the recognized inadequacies of the "M1/M2" nomenclature, it has been postulated that a continuum of activity states exists between the polarized extremes, resulting in studies presenting "M1" subtypes to better represent the heterogenic nature of these reactive cells[87,88]. Recent studies however, have shown that factors assigned to either the "M1" or "M2" phenotype are promiscuous yielding low fidelity to their assigned reactive state[88-90]. Thus, the complexity of microglia function is undermined by the overly simplistic and polarized naming scheme of "M1/M2".

Disease-associated microglia

Another subclass of reactive microglia that is specific to non-homeostatic conditions is known as Disease-Associated Microglia (DAM). First identified in Alzheimer's disease and amyotrophic lateral sclerosis models[91], DAM or microglia with DAM-like phenotypes have now been described in tauopathy models[92,93] multiple sclerosis[94] and aging[91,95]. DAM express typical microglia markers including Iba-1,

cystain 3 and hexosaminidase subunit beta. DAM can downregulate homeostatic genes including *P2ry12*, *Cx3Cr1*, and transmembrane protein 119 (Tmem119), and upregulate genes in either a triggering receptor expressed on myeloid cells 2 (Trem2) dependent (Axl, C-type lectin domain containing 7A, secreted phosphoprotein 1) or independent (Apolipoprotein E, TYRO protein tyrosine kinase-binding protein) manner[91]. Interestingly, another related class of microglia, which present with a similar expression profile as DAM[94], was recently described and named microglial degenerative phenotype (MGnD). It remains to be determined if MGnD and DAM represent the same subclass of cells.

Although unique to non-homeostatic conditions, the function of DAM is not known. It has been hypothesized that these cells respond to a CNS stress signaling system that is akin to the peripheral immune system's pathogen- and damage-associated stress signals (PAMPs and DAMPs)[96]. In this scenario, danger signals are recognized by microglia and trigger the transition of surveying microglia into DAM. This hypothesis is consistent with DAM accumulation in Alzheimer's Disease plaques and regions of demyelination[91,94,97,98]. If correct, DAM would be a key component of an intrinsic mechanism designed to combat disease processes and could provide a promising target for therapeutic manipulation against neurodegenerative disease by further enhancing the DAM response.

Heterogenic expression in inflammatory microglia

Following injury or disease, reactive microglia are rapidly recruited to sites of damage where they phagocytose debris and dying cells, consistent with the described functions of DAM. Likewise, AXIS microglia may also be recruited to sites of damage following injury or disease[80,81]. However, unlike DAM, the expression profile of AXIS microglia has not been characterized. Instead, AXIS microglia have been characterized based on their physical interactions with the axonal domain of the AIS. Both surveying and reactive microglia make contact with AISs and this is increased or decreased based on the disease context[78,79]. Whether these cells provide trophic support at the AIS or drive pathogenesis remains unclear though. Reactive microglia also exhibit extensive changes in expression of their inflammatory profile[99]. While some of these secreted factors may provide neurotrophic functions, pro-inflammatory factors can also exhibit deleterious effects[100,101]. For example, pro-inflammatory microglia ("M1") upregulate enzymes that produce reactive oxygen species (ROS)[100]. Activation of microglial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) results in the extracellular production of ROS[102]. ROS then alter the function of calcium-permeable ion channels[103-105] and consequently, alters intracellular calcium levels[105,106], which have been implicated in AIS disruption[81,107-110].

In addition to regulating neuronal function through secreted factors, microglia also regulate neurons through physical contact[82,111-115]. In the developing and adult brain, microglia contact pre- and postsynaptic neuronal elements in an activity-dependent manner, and synapses that are contacted more frequently are subsequently removed[17,115,116]. In pathological conditions, microglia participate in synaptic stripping altering the neuronal excitatory/inhibitory balance[16]. Microglia also preferentially contact cell bodies and axons of highly active neurons to decrease neuronal activity and prevent excitotoxic cell death[113,114]. These studies underscore the importance of microglial contact in the regulation of neural signaling.

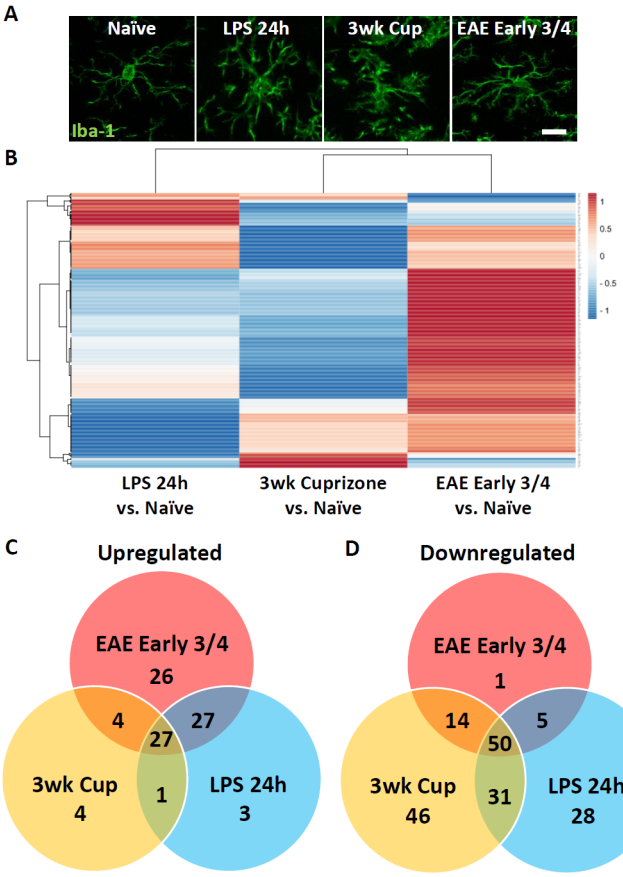
Recently, we analyzed CNS pathology in three models of neuroinflammation. In all three models, microglia presented with reactive phenotypes and these cells maintained, or even increased, contact with the AIS. However, in two of the models, the AISs were disrupted and in one, the AISs were preserved. Since AIS integrity temporally correlated with the presence of reactive microglia and contact was at least maintained in all three models, we proposed that differential AIS integrity was consequential to the heterogeneity among the reactive microglia from all three models.

For our studies, we exploited the immune-mediated inflammatory models of EAE[79], LPS[80] and the demyelinating model of cuprizone[79]. The EAE model is induced through subcutaneous injection of

myelin peptide (myelin oligodendrocyte glycoprotein peptide 35-55) accompanied by pertussis toxin and an adjuvant to ignite an inflammatory response[117-119], which transfers to the brain and results in chronic neuroinflammation persisting for months. While neuroinflammation is present after induction and throughout the EAE course, clinical symptoms do not begin to appear until ~15 days post-induction[79]. Mice exhibit a range of clinical symptoms from limp tail and loss of righting reflex (EAE 1 & 2, respectively) to single- or double- hind limb paralysis (EAE 3 & 4, respectively)[79]. AIS pathology begins to appear at an early timepoint after clinical onset (~18 days post-induction), but only in mice that display more severe clinical symptoms (EAE Early 3 & 4). Increased AIS pathology is observed with disease severity and progression (EAE Late 1 & 2, 3 & 4, ~25 days post-induction)[79]. In contrast, the LPS model is an acute neuroinflammatory model induced by a single peripheral injection of LPS[120,121]. This results in widespread peripheral inflammation that rapidly transfers to the brain (~3 h), but the neuroinflammation is resolved by 2 weeks post-injection. In the LPS model, AIS pathology was present from as early as 24 h post-injection and persisted until 1 week post-injection, coincident with the initiation and resolution of the acute neuroinflammatory environment[80]. In contrast to the immune-mediated neuroinflammatory models, the cuprizone model is a demyelinating model[79] where a copper-chelating toxin, cuprizone, is administered through chow resulting in oligodendrocyte cell death and, consequently, loss of myelin[119]. Demyelination is detectable 1-2 weeks after cuprizone treatment with peak demyelination occurring by 5-6 weeks of exposure[122-124]. The cuprizone model yields substantial cell death and demyelination resulting in microglial recruitment and neuroinflammation but no AIS pathology was observed[79].

We utilized these three models to further investigate microglial heterogeneity. AIS disruption only occurred in the LPS and EAE models, while microglial-AIS contact was abundant in all three models. Thus, while microglial reactivity and contact increased prior to and was coincident with disruption in EAE, contact alone did not disrupt AIS integrity[79,80]. Therefore, we analyzed the inflammatory expression profiles of cortical microglia across all three models to assess how microglial reactivity differentially influences neuronal integrity. Our goal was to assess microglia expression profiles early in the disease process to identify inflammatory changes that drive disease progression and are not consequential of disease progression. Thus, cortical microglia were isolated from mice induced with EAE, Cuprizone, or LPS at time points where neuronal pathology is detectable but had not peaked (EAE Early 3 & 4[79], 3 week Cuprizone[123], LPS 24 h)[80]. Briefly, total RNA, collected from cluster of differentiation (CD) 11b$^+$ cells isolated from the cortex of c57black6 female mice, was submitted for NanoString mRNA expression analysis. (Further details on model generation, cell isolation and NanoString analyses are provided in Supplementary Materials[79,80,120-123,125-133]). Cells were collected at time points in each model that corresponded to the early presence of neuronal/myelin pathology, but prior to peak disease course in an effort to understand the inflammatory profiles that drive pathogenesis[79,80].

Microglia with reactive morphologies predominate in the cortex of all three models[79,80] [Figure 2A], which is consistent with these cells presenting with a pro-inflammatory phenotype. However, based on NanoString expression analysis of 248 inflammation-associated genes, microglia from all three neuroinflammatory models displayed distinct regulation of inflammatory genes [Figure 2B], underscoring the heterogeneity of morphologically similar cells. Of 248 analyzed genes, 95 were significantly upregulated (1.3 fold-change or greater) [Figure 2C] and 175 were significantly downregulated (at least 1.3 fold-change) among the three neuroinflammatory models when compared to microglia from naïve mice [Figure 2D]. 27 of 95 (28.4%) upregulated genes [Figure 2C] and 50 of 175 (28.6%) downregulated genes [Figure 2D] were similarly changed across all three models but model-specific differences were observed for both categories. Numerous genes [Figure 2C] associated with a pro-inflammatory ("M1") phenotype (such as interferon regulatory factor 1, lymphotoxin beta, C-C chemokine receptor type 7, C-C motif chemokine ligand 7, C-C motif chemokine ligand 17, lymphotoxin Alpha, Il1a, signal transducer and activator of transcription 2, and tumor necrosis factor super family 14) were upregulated uniquely in EAE Early 3 & 4 and LPS 24 h



E

Group	# of Genes								
EAE total	86 ↑ 70 ↓	EAE only	26 ↑ 1 ↓	EAE and Cuprizone total overlap	33 ↑ 64 ↓	EAE and Cuprizone only overlap	4 ↑ 14 ↓	All overlap	27 ↑ 50 ↓
Cuprizone total	39 ↑ 141 ↓	Cuprizone only	4 ↑ 46 ↓	EAE and LPS total overlap	56 ↑ 55 ↓	EAE and LPS only overlap	27 ↑ 5 ↓		
LPS total	60 ↑ 114 ↓	LPS only	3 ↑ 28 ↓	Cuprizone and LPS total overlap	30 ↑ 81 ↓	Cuprizone and LPS only overlap	1 ↑ 31 ↓		

Figure 2. Microglia differentially express inflammation-associated genes in three neuroinflammatory models that demonstrate robust microglial reactivity. A: representative images of surveying microglia from the cortex of naïve mice and reactive microglia from LPS 24 h, 3 week Cuprizone, and EAE Early 3 & 4 mice; B-D: analysis of NanoString data of 248 differentially expressed inflammation-associated genes in CD11b$^+$ cells. Background subtraction was performed using the maximum value across samples of the negative controls and data normalization was performed using the geometric mean expression of six internal reference genes (*CLTC*, *GAPDH*, *Gusb*, *Hprt*, *Pgk1*, *Tubb5*). Reporter probe counts reflecting the numbers of mRNA transcript in the RNA sample were analyzed and quantified using the nSolverTM Analysis Software, and are represented by fold-change compared to naïve cells. Two mice were pooled per sample and three total samples per group were submitted for NanoString analysis. Microglia were isolated by CD11b Miltenyi beads from the cortex of mice induced with EAE, Cuprizone, or LPS at early time points where neuronal pathology was detectable but had not peaked (EAE Early 3 & 4[79], 3 week Cuprizone[123], LPS 24 h)[80]; B: heat map of differentially expressed genes; C: venn diagram representing the number of genes that are significantly upregulated, 1.3 fold-change or greater, in microglia from mice induced with EAE, Cuprizone, or LPS; D: venn diagram representing the number of genes that are significantly downregulated, 1.3 fold-change or greater, in microglia from mice induced with EAE, Cuprizone, or LPS; E: table showing the number of genes that were significantly upregulated (upward arrow) or downregulated (downward arrow) in each experimental group, and the number of altered genes shared among groups. Scale bar = 10 μm. $P < 0.05$. LPS: lipopolysaccharide; EAE: experimental autoimmune encephalomyelitis

mice, consistent with the involvement of infective and inflammatory response pathways[127]. Gene ontology biological processes (GO-BP) function analysis[125,126] revealed that these genes were involved in functions related to regulation of the pro-inflammatory response as defined by the production of tumor necrosis factor alpha, nitric oxide biosynthetic process, and chemotaxis and chemokine signaling. In contrast,

microglia from 3 week cuprizone treated mice had the greatest number of downregulated inflammatory genes, and the top four uniquely upregulated genes associated with phagocytosis and oligodendrocyte generation [guanine nucleotide-binding protein G(s) subunit, platelet derived growth factor alpha, TYRO protein tyrosine kinase-binding protein (TyroBP), C-C chemokine receptor type]. Platelet derived growth factor alpha is a mitogen that is critical for oligodendrocyte generation[134]. TyroBP is a microglial transmembrane signaling polypeptide that forms phagocytosis active zones preparing microglia for phagocytic activity[135]. Increased expression of TyroBP in the 3 week treated cuprizone mice, a treatment time point that corresponds to early myelin loss, is consistent with findings from transcriptome microglial analysis from demyelinating regions in other animal studies[136] and human tissue[137]. Thus, while microglia from all three models exhibit pro-inflammatory ("M1"), reactive expression profiles, microglia maintained a unique "fingerprint" for each model and these differences correspond with the integrity of the AIS, suggesting that subtle changes in microglial phenotype may mediate either stability or disruption of closely apposed neurons. It is still possible though that microglial phenotypes do not directly influence AIS integrity. The direct association of microglia with the AIS suggests however, that this neuronal domain may be particularly vulnerable to changes in microglial reactivity. These data support the growing body of literature demonstrating that microglia exhibit a plethora of inflammatory expression profiles within an "M1" phenotype despite having similar morphologies.

Transcriptomic defined subsets of microglia

Recently, several single cell RNA sequencing studies have begun to more clearly define subsets of microglia in the developing,mature and healthy, and pathologic CNS[45,138,139]. Grabert and colleagues[45] conducted the first genome-wide comparison of RNA expression profiles from microglia isolated from specific brain regions and across the adult life span. Their findings confirmed the presence of core profiles that distinguish microglia from macrophages, underscoring their distinct origins. In addition, they observed three primary RNA profiles that were regionally specific, demonstrating regional heterogeneity within the microglial population. Although regional specific heterogeneity was observed, similarities persisted between the cortex and the striatum, and between the cerebellum and hippocampus. With age, some of these differences dissipated as the profile of hippocampal microglia appeared to converge with the profiles of microglia from the cortex and striatum, while the profile of cerebellar microglia continued to diverge from the other three regions to reveal region specific changes over time. Li *et al.*[138] reported that the majority of microglia in mature, healthy CNS express similar profiles but significantly greater diversity was seen in postnatal CNS. An interesting finding of Li *et al.*[138] was the similarity between a postnatal subset of microglia, termed Proliferative-region Associated Microglia (PAM), and DAM, which demonstrates that genes expressed in development are reactivated with aging and pathology. PAM appeared transiently in regions of developing white matter, consistent with a role in phagocytosing the large numbers of oligodendrocytes that die during myelination[140]. The authors further state that the complete chemokine and cytokine expression profile of PAM supports additional roles including interacting with both neural and immune cells.

Using fluorescent assisted cell sorting gated by CD11b, CD45 and Cx3Cr1, Hammond *et al.*[139] defined nine unique clusters of microglia in the whole brain based on expression profiles. The percent of cells in each cluster changed across age and condition however. Canonical microglia genes were expressed by most cells but only *C1qa*, *Fcrls* and *Trem2* were expressed in all clusters. Interestingly, P2ry12, Cx3Cr1 and Tmem119, which are frequently used as microglial identifiers[141-143], were either expressed in very low levels, or not at all in some clusters during development. Additionally, a novel subset of microglia, defined by the expression of secreted phosphoprotein 1, similar to PAM described by Li *et al.*[138], insulin like growth factor 1 and immunomodulators from the galectin family and several lysosomal proteins, was observed in the postnatal brain and associated with axonal tracts destined for myelination. Since these microglia express lysosomal markers, it was proposed that these cells clear the way for continued axon outgrowth, ultimately facilitating subsequent myelination. Other interesting findings include the lack of sex differences

based on cluster comparisons, which is in contrast to previous reports[48,50,54,128]. Although sex differences were not observed, significant differences were observed within the aged brain (postnatal day 540) as certain clusters, which were comprised of very few cells in the adult brain (postnatal day 100), revealed a significant increase in the number of cells in the aged brain. Perhaps most interesting is the finding that specific subpopulations of microglia were similarly represented in demyelinating lesions in the mouse and human brains, suggesting that microglial cluster expression profiles may allow identifying disease-specific-"fingerprints", and eventually aid in human disease treatment.

CONCLUSION

Although described 100 years ago, we are only just beginning to put together the various pieces of the microglial puzzle. We now recognize their involvement in establishing and maintaining a homeostatic CNS environment through trophic support and pruning of both neuronal and glial populations, modulating CNS wiring and circuitry, and facilitating axonal organization and outgrowth, myelin formation, and immunosurveillance in the healthy brain. Moreover, we are also beginning to appreciate their critical roles in disease, potentially both as CNS protectors by recognizing and removing infected, dying and dead cells, and also as CNS villains secondary to hyperactivation or dysregulation. We are also beginning to recognize that microglia may present as functionally distinct subclasses, which provides an explanation as to how a single lineage cell type can manifest into a plethora of diverse roles. However, it remains to be determined if distinct subclasses of microglia truly exist, or if microglia exist on a spectrum where they have the capacity to take on a multitude of identities depending on their environment. To address this issue, consistent approaches in cell isolation and analysis should be established and implemented. Additionally, as presented by other authors[86], the generation of a naming scheme that incorporates all aspects (age, brain region, morphology, gene expression, function, *etc.*) of microglia is essential for effectively moving the field forward. Although much has been learned over the past 20 years, our understanding of microglia remains limited. The immediate future though should be viewed with excitement as we continue to unravel the mysteries of these enigmatic cells.

DECLARATIONS

Authors' contributions

Made substantial contributions to experimental conception and design and manuscript preparation: Dupree JL

Made substantial contributions to experimental conception and design, in technical support, mRNA data analysis and interpretation and manuscript preparation: Benusa SD

Made substantial contributions to microglia-AIS contact analysis and interpretation: George NM

Availability of data and materials

NanoString raw data files are provided in Supplementary Material.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Resolution of inflammation and repair after ischemic brain injury

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Abstract

After ischemic stroke, proinflammatory molecules known as danger-associated molecular patterns (DAMPs) originating from damaged brain cells recruit and activate immune cells (neutrophils, macrophages, lymphocytes) further eliciting innate and adaptive immunity. During the acute phase from day 1 to day 3 of the stroke onset, macrophages play a major role in the progression of inflammation, promoting the destruction of brain tissue. During the recovery phase, from day 3~4 to day 7 after stroke onset, infiltrating macrophages switch to repairing macrophages, which clear the DAMPs and promote tissue repair by producing neurotrophic factors. Adaptive immunity during the late or chronic phase (> day 7) of stroke has not been well investigated. Recent studies have also indicated that antigen-specific T cells, especially regulatory T cells (Tregs), play major roles in neural repair. This review focuses mainly on the resolution of inflammation and tissue repair by macrophages and Tregs.

Keywords: DAMPs, tissue repair, macrophages regulatory T cells, amphiregulin, IL-33

INTRODUCTION

Ischemic cerebral infarction accounts for 70% to 80% of all strokes, which is the leading cause of severe neuropathy, disability and bedriddenness[1]. Ischemic stroke causes the death of nerve cells as well as destruction of neuronal circuits, which leads to movement disorders, higher brain dysfunction, and sensory

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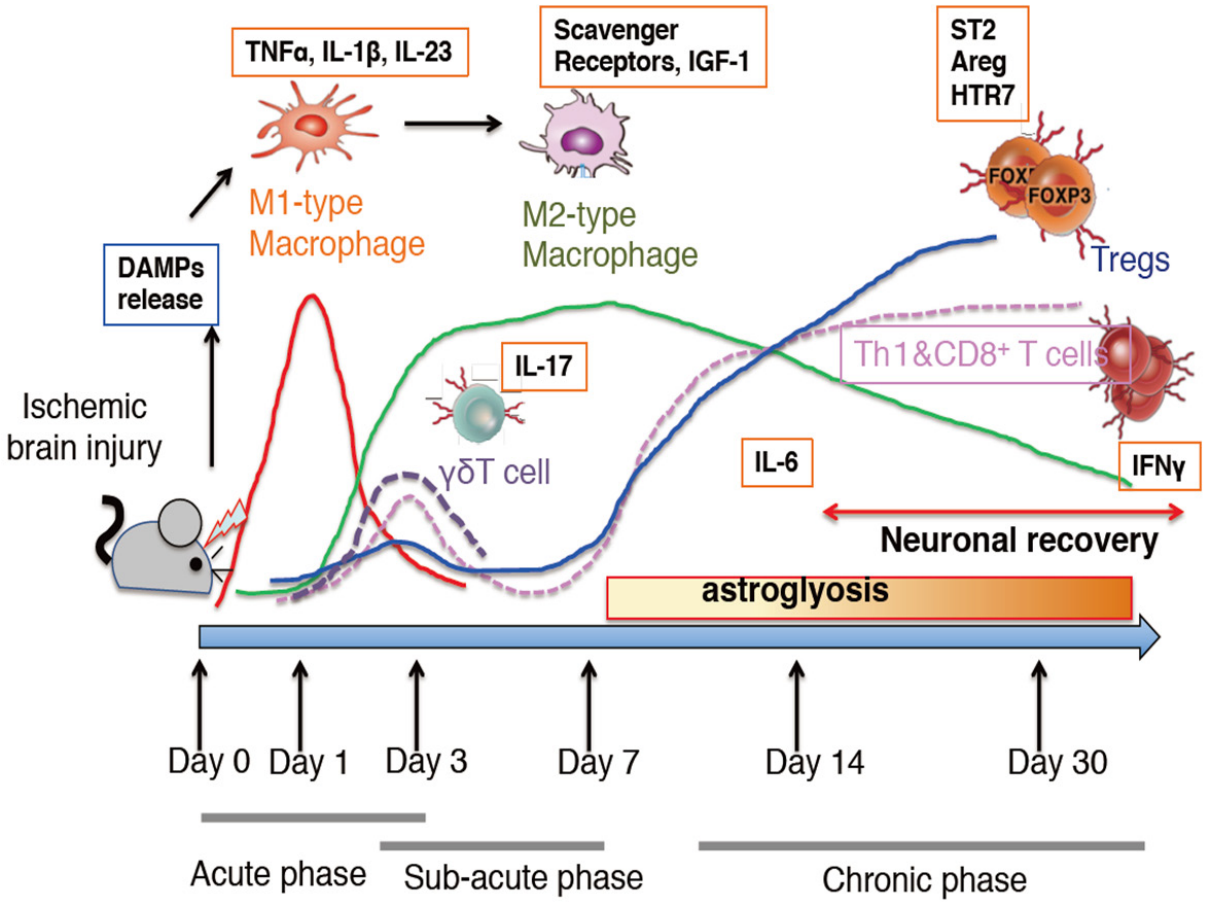

Figure 1. Schematic viewing a time-dependent recruitment of various inflammatory cells into the brain following cerebral ischemia in mice. In this review, we refer to day 1-3 after stroke onset as the acute phase, for days 3-7 being subacute phase, and period after 2 weeks being chronic phase. This figure illustrates the conceptual changes in the population of immune cells, thus the numbers of each immune cell may not necessarily be accurate. Red line; M1 type inflammatory macrophages, green line; M2 type macrophages, gray broken line; γδT cells, pink broken line; Th1 cells and $CD8^+$ T cells, blue line; regulatory T cells (Tregs). IL: interleukin; IGF: insulin growth factor

disturbance. Ischemic damages of the brain tissue further induce cerebral edema and inflammation, which exacerbates the functional prognosis and symptoms of stroke. Although administration of tissue-plasminogen activator reduces ischemic neural damages, this treatment should be instituted within 4.5 h of stroke onset[2]. After this acute stage, no therapeutic drugs should be administered during the late stage of stroke, and following this short period, rehabilitation is the only modality of treatment for functional recovery at present.

Sterile inflammation initially leads to tissue damages[3]. Likewise, in ischemic stroke, brain inflammation causes neural cell death and has been considered to be an attractive target for reducing brain damages not only in experimental rodent models but also in human patients[4-6]. As inflammation occurs within a few days after stroke onset, innate immunity, in which microglia, macrophages, neutrophils, and γδT cells play major role, has been thought to account for neuroinflammation after stroke, where such inflammation disappears after 1 week of stroke onset [Figure 1]. To date, only a small number of studies have investigated the adaptive immunity in stroke, which usually occurs over a week after the onset of the disease or infection[7,8]. However, our group and others have discovered an accumulation of lymphocytes including regulatory T cells (Tregs) in the brain at the chronic phase (more than 2 weeks) after stroke onset. This process has been shown to be involved in neural repair rather than progression of the disease[9-13].

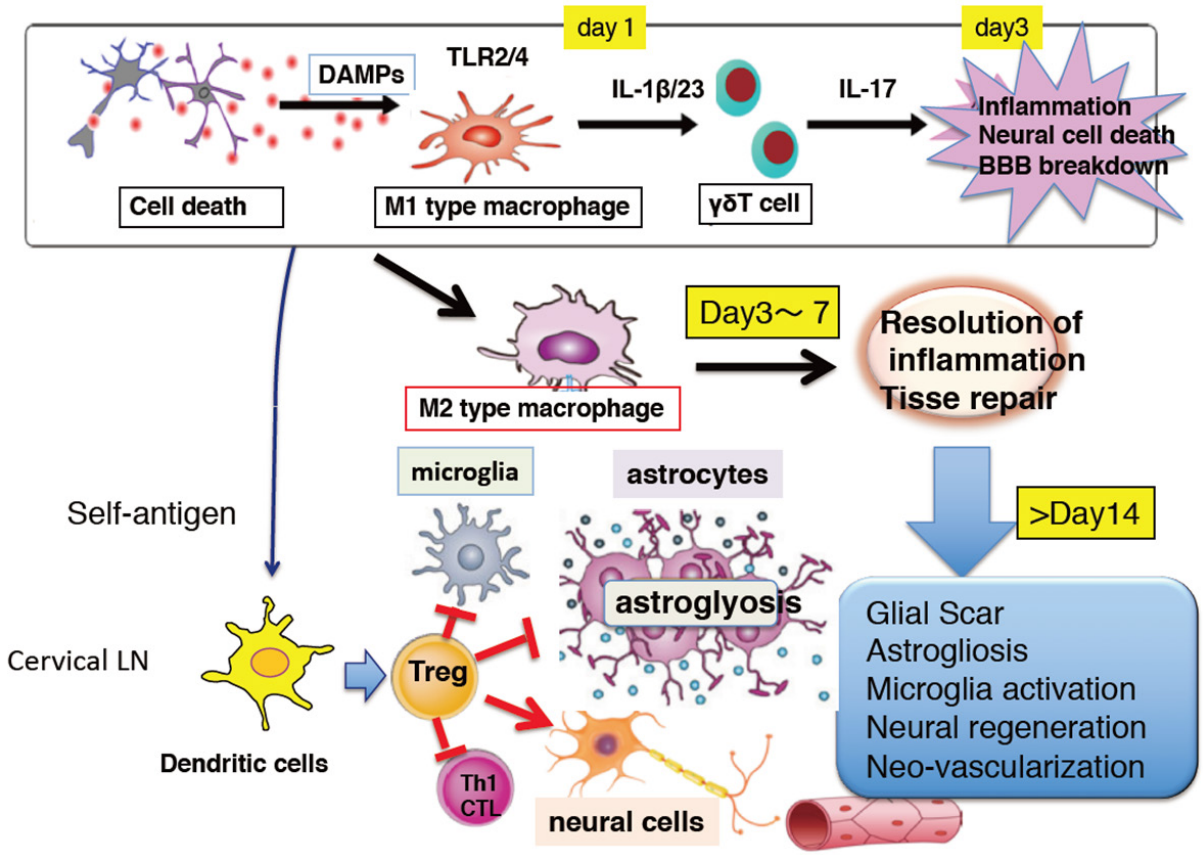

Figure 2. Schematic view of the role of immune cell types in cytokine production, neuroinflammation, and tissue repair. In the early stage of around 24 h, macrophages infiltrate the injured brain and are activated through the TLR2/4 stimulation by molecules known as danger-associated molecular patterns (DAMPs) from necrotic cells. Thereafter, infiltrated macrophages produce inflammatory cytokines and mediators which increase probability of ischemic encephalopathy and blood brain barrier (BBB) destruction. At this stage, macrophages become M1-type inflammatory macrophages. Further on, IL-23 and IL-1β from inflammatory macrophages stimulate IL-17 production from infiltrated γδT cells. After day 3, macrophages are converted into M2-type repairing macrophages which are involved in clearing DAMPs, resolving inflammation, and tissue repair by producing neurotrophic factors such as IGF-1. During the chronic phase, a massive T-cell infiltration occurs. Brain Tregs are attracted via chemokines and proliferate in the cervical lymph node (LN) and the brain. Brain Tregs may thereafter interact with various brain cells including that of microglia, astrocytes, endothelial cells, and neural cells promoting neural cell recovery in th process. IL: interleukin; IGF: insulin growth factor

In this mini-review article, we will focus on the resolution of inflammation and tissue repair of the brain after ischemic stroke.

INFLAMMATION CASCADE AFTER STROKE

In the very acute stage (< 24 h) after stroke onset, the major player involved is infiltrated macrophage, which can be differentiated from bone marrow-derived monocytes. Macrophages are activated by extracellular molecules known as danger-associated molecular patterns (DAMPs), which are released from damaged and dead cells. Infiltrated macrophages at this stage are highly pro-inflammatory and produce cytokines, chemokines and mediators which exacerbate ischemic encephalopathy, leading to dysfunction of the blood brain barrier (BBB)[4,14] [Figures 1 and 2]. HMGB1, S100A8 and S100A9, and peroxiredoxin-family proteins are major DAMPs which activate infiltrated macrophages through toll-like receptor (TLR)-2 and TLR-4[15].

Among inflammatory cytokines released from macrophages, TNFα, interleukin (IL)-1β, IL-23 have been shown to contribute significantly to brain damages and neural dysfunctions[16-19]. In particular, IL-1β and

IL-23 induce IL-17 production from infiltrated γδT cells, further promote inflammation, BBB breakdown, and neuronal damage[19-21]. IL-17 is a cytokine which has been shown to play important pathological roles not only in ischemic stroke, but also in various neuroinflammation including neurodegenerative diseases[22-24]. The inflammasomes, which are necessary for mature IL-1β release, have also been described to cause deterioration in infarct volume resulting in neural defects in stroke patients. Inhibition of inflammasome is shown to be effective for reducing neuroinflammation and subsequently reducing infarct volume increase[20,25].

RESOLUTION MECHANISM OF INFLAMMATION AFTER STROKE

After 3-4 days post-ischemic stroke onset, inflammatory macrophages termed M1 type macrophages, are converted into repairing macrophages or M2-type macrophages. Repairing macrophages play a role in scavanging tissue debris and necrotic cells, futher supporting neural repair by releasing neurotrophic factors including insulin growth factor (IGF)-I[26] [Figure 2]. It has been suggested by some studies that IL-10 and TGF-β from M2-type macrophages and microglia, promoting the resolution of inflammation[27]. Is is not clear whether the same M1 macrophages may convert to M2 type, or M2 macrophages replace M1 type macrophages although an imaging study of infiltrated macrophages in experimental autoimmune encephalomyelitis (EAE) model revealed that single macrophage changes its phenotype from M1 to M2[28]. For clearance of DAMPs, Msr1 (macrophage scavenger receptor-1 or what is known as CD204 or SCARA1) was identified as a major scavanger receptor[29,30]. *Msr1* promoter was described to be activated by *Maf-b*, and an RAR agonist, Am80, upregulating MAF-B expression, therefore promoting Msr1 expression and clearance of DAMPs which ultimately facilitates neurological recovery[29]. Am80 has also been shown to be neuroprotective by activating the PI3-kinase/Akt pathway[31]. Msr1 has been shown in several studies to clear various neurotoxic molecules including amyloid-β thus playing an important neuroprotective role[32,33]. Mannose receptors on infiltrating macrophages have also been reported to be involved in the clearance of DAMPs in focal cortical ischemia[34].

The early activation of microglia in the post-ischemic brain was demonstrated to be neuroprotective by regulating neuronal Ca^{2+} overload and spread of depolarization. Pharmacological ablation of microglia results in infract size increase and dysregulation of neuronal circuit, while microglia repopulation reverses these effects[35]. The pro-resolving mediators including protectins and resolvins, which have been shown to be neuroprotective. Resolvins reduce neural damage through suppression of leukocyte infiltration, IL-1β expression, and NF-κB activation[36]. Neuroprotectin-D1 similarly reduces infarct volume and diminishes disease burden[37]. LXA_4 had also been reported to be neuroprotective by virtue of mitigating astrogliosis, IL-1β, TNFα expression, and neutrophil infiltration. Additionally, it also converts phenotypes of monocytes from inflammatory to an anti-inflammatory and serves functionally to repair tissues[38]. However, the mechanism of resolution (or suppression) of inflammation by microglia and these lipid-mediators remains to be described.

Lack of CCR5 expression has been reported to increase the severity of ischemic brain injury[39]. CCR5 is uniquely expressed in cortical neurons within the damaged brain[40]. CCR5 antagonists accelerate recovery from neurological and cognitive dysfunction. Although various roles of CCR5 in neurons have been reported, the inhibition of CCR5 has been found to suppress astrocyte reactivity and macrophage recruitment[40,41]. Nevertheless, another study has revealed the pathogenic role of CCR5 in cerebral ischemia[42], suggesting that various types of cells may express CCR5 and contribute to both neuronal inflammation and tissue repair of the ischemic brain.

ROLE OF MICROGLIA IN RESOLUTION OF INFLAMMATION AND NEURAL REPAIR

Microglia have been shown to play important roles in neural inflammation, resolution of inflammation and clearance of dead cells in the brain[43]. Since major sources of IL-1β and IL-23 is infiltrated M1 type

macrophages originate during the acute phase of inflammation after stroke model[14,20], the contribution of microglia to severe inflammation post stroke is not very clear. Depletion of microglia leads to aggravated neuronal damage and apoptosis after ischemic brain injury, suggesting that microglia plays an important role in neuroprotection[44]. Mechanistically, microglia senses damaged cells through the purinergic receptors (P2X4R, P2X6R, P2X12R) which respond to adenosine triphosphate released from dead cells. The activation of the purinergic receptors initiates neuroprotective responses rather than destructive inflammation through the recruitment of microglia to the point of injury[45,46]. Microglia also express clearance receptors such as Msr1, complement receptors, and receptors for apoptotic cells[32,47]. A recent study shows beneficial effects of repopulating microglia which support adult neurogenesis by augmenting the survival of newborn neurons in traumatic brain injury thorough IL-6 trans-signaling pathways[48]. IGF-1 produced by microglia has been found to be neuroprotective[49]. However, the significance of IGF-1 in neuroinflammation and neuroprotectin remain controversial[50].

ROLE OF LYMPHOCYTES IN POST-ISCHEMIC BRAIN INFLAMMATION

It has been reported that lymphocytes including T cells and B cells play various roles in the pathophysiology of stroke[51]. B cells and immunoglobulins are detected within and around the stroke core in a subgroup of stroke and dementia patients, and also in a murine experimental stroke model. Several studies suggest that post-stroke cognitive impairment has been associated with B cell activation and auto-antibody production[52]. Nevertheless, the specific roles of B cells and/or antibodies in neurological deficits and inflammation after ischemic brain injury remain uncertain[53]. Plasma cells in the central nervous system (CNS) of mice with EAE have been shown to originate in the gut and produce IgA, which confers resistance to mice to the effector stage of EAE through the production of IL-10[54]. Interestingly, stroke patients demonstrate a type of auto-immunoreactivity to brain antigens[55].

T cells have been more intensively investigated than B cells. This is mostly because various cytokines, such as IL-10, IL-17, IL-21, IFN-γ, and TNF-α produced from $CD4^+$T cells and/or γδT cells, affect and regulate glial cells, endothelial cells, neural cells, and various immune cells[56,57]. IL-21 is predominantly produced from $CD4^+$T cells, but the role of this particular cytokine in stroke is controversial. In mice, a locus on distal chromosome 7 has been described to contribute variations in post-ischemic cerebral infarct volume, and the IL-21 receptor has been identified as a strong candidate which functions in a neuroprotective manner[58]. However, another study suggested that IL-21 promotes brain injury after stroke in mice[57], thus further research is necessary to clarify the role of IL-21 in brain injury.

The route of infiltration of T cells to the brain is not described clearly. A recent study shows that T cells specifically accumulate within the peri-infarct cortex after stroke and that the ipsilateral choroid plexus plays a key cerebral invasion route for T cells[59]. This study suggests that the CCR2-ligand gradient between cortex and choroid plexus serves as the potential driving force for T cell invasion.

In the experimental cerebral ischemia model, infiltration of subsets of T cells occurs at various time points [Figures 1 and 2]. Many reports indicate that T cells promote brain damage at the early phase of stroke[19,60]. In humans, FTY720 (fingolimod) treatment within 72 h post stroke onset blocks the infiltration of pathogenic T cells into the brain, effectively ameliorating neurological symptoms in the patient[61]. $CD8^+$ T cells may infiltrate within several hours after stroke onset[61]. Nerve-damaging substances such as granzymes and perforin from $CD8^+$T cells can exacerbate the infarction[62]. γδT cells increase immediately after stroke onset and are present in the brain parenchyma accompanied with BBB breakdown. On day 3 after stroke onset, the number of γδT cells reaches its maximum concentrations[19,21]. IL-17 produced from γδT cells promotes neural cell damages in the ischemic penumbra region[19,21,63]. Taken together, γδT cells depletion well as anti-IL-17 neutralizing antibody are shown to suppress ischemic brain injury[19,64]. $CD4^+$T cells and NKT cells infiltrate the brain after 24 h of ischemic stroke[65]. These reports indicate that

T cell could serve as a therapeutic target for stroke. However, we should remain cautious that within one week after stroke onset, the number of T cells in the brain is small and represents only a small fraction of infiltrated mononuclear cells[19] [Figure 1].

It has been reported that during the acute phase of stroke, Tregs infiltrate the brain, suppressing neuroinflammation, therby reducing the severity of ischemic brain injury[60,66,67]. However, the significance of Tregs in brain injury has become controversial[68,69]. Importantly, the number of Tregs in the brain at this stage is extremely low (less than 100 cells/brain in mice), and antigen-specific activation and proliferation of Tregs may not occur in such a short period (within 3 days), since it usually takes more than a week to raise adaptive immunity in the host [Figures 1 and 2]. Thus, bystander effects, such as paracrine effects of IL-10, may explain an anti-inflammatory role of Tregs at the acute phase.

ACCUMULATION OF BRAIN TREGS AT THE CHRONIC PHASE OF STROKE

It is thought that inflammation no longer plays an important role in neural damage and recovery at the chronic phase of stroke (> 7 days after stroke onset). Inflammation is not clearly obvious at this stage. However, compared to the acute phase, Tregs as well as other lymphocytes have been shown to accumulate in substantial quantities in the brain at the chronic phase of the experimental stroke model[9-13] [Figures 1 and 2]. Infiltration of Tregs proceeds with slightly delayed kinetics compared with that of other T cells. Tregs may also infiltrate the spinal cord parenchyma during the subacute to chronic phases in the spinal cord injury model[70]. Tregs consist of approximately 50% of $CD4^+$ T cells, and localize within and around the cerebral infarction lesion. Outside the infarct core area, Tregs remain in close proximity to scar-forming astrocytes and neuronal cells. Treg fractions in the brain are extremely higher that those in other lymphoid organs such as the spleen and lymph nodes. Since $CD8^+$ T cells are also present, about 1/4 to 1/5 of T cells in the brain are calculated as Tregs.

To determine the role of T cells in ischemic brain injury, mice were treated with FTY720 or anti-CD4 antibody during the chronic phase after stroke. Tregs can also be depleted by the use of Foxp3-diphtheria toxin receptor (DTR) mice, where DTR is specifically expressed on Tregs[71]. These treatments drastically reduce the number of $CD4^+$T cells including Tregs in the brain, delaying neurological recovery. These data indicate that brain Tregs at the chronic phase are important for suppressing neurological symptoms[9]. Stubbe *et al.*[10] observed no changes regarding neurologic outcome if they depleted Tregs through the use of anti-CD25 antibody[10]. Anti-CD25 antibody, however, may not be able to completely deplete Tregs, possibly depleting pathogenic T cells as well[72]. Other studies have also shown that brain Tregs play neuroprotective roles during the late stage of stroke and spinal cord injury models[12,73].

CHARACTERIZATION OF BRAIN TREGS

Tregs consist of approximately 10% of $CD4^+$ T cells, located within most lymphoid organs and blood, moving to specific sites of inflammation after immunization. In addition, Tregs have recently been discovered in various tissues besides lymphoid tissues, in steady state conditions as well as during injury. These tissue-residing Tregs are now termed "tissue Tregs", which have a limited TCR repertoire and recognize the self-antigen characteristically expressed in each tissue. Such tissue Tregs exist in fats, muscles, skin, lungs, and intestines, exhibiting similar phenotypes among organs, but are quite different from those of lymphoid tissue[74-78]. The features common to various tissue Tregs are high expressions of *Il10*, *Areg* (amphiregulin), *Klrg1*, *Tigit*, *Il1rl1* (encoding ST2, IL-33 receptor), *Ctla4*, *Irf4*, *Batf*, and *Gata3* and low expressions of *Bcl2*, *Tcf7*, and *Lef1* compared with lymphatic Tregs[74,79]. BATF is shown to be an important regulator for Tregs to accumulate preferentially in several tissues[80]. In addition to the common genes expressed in various tissue Tregs, unique tissue-specific genes are also found in tissue Tregs namely *Pparg* in fat Tregs. The microenvironment of each organ appears to determine the tissue-specific phenotypes of tissue Tregs.

Like other tissue Tregs, brain Tregs express Helios, which suggests that brain Tregs are derived from the thymus embryonically. Brain Tregs possess a unique TCR repertoire and express high levels of CTLA-4, PD-1, Areg, KLRG1, and ST2, indicating that the brain Tregs share common features of tissue Tregs.

MOLECULES DEEPLY INVOLVED IN TREG-MEDIATED NEURAL RECOVERY

Chemokine receptors

Tissue Tregs express unique chemokine receptors in each organ. Brain Tregs express specific chemokine receptors including CCR6 and CCR8, and their ligands. CCL20 and CCL1 in particular are highly expressed in the cerebral infarct area. Intra-ventricular injection of CCL1 and CCL20 has been reported to increase the number of Tregs, resulting in improvement of neurological recovery[9].

IL-33

Of note, IL-33 promotes tissue recovery after CNS injury[81] and up-regulates M2 type macrophage-related genes[82]. Since many reports suggest that IL-33 induces the expansion of Tregs in the brain[9,12,83], it is highly possible that brain Tregs are involved in the beneficial effects on CNS damage[12,84]. In the skeletal muscle injury model, local mesenchymal stromal cells express the receptor for the calcitonin-gene-related peptide (CGRP), producing IL-33 in response to CGRP, which further promotes accumulation of Tregs and muscle tissue repair[78]. IL-33-expressing cells in the brain consist of astrocytes and oligodendrocytes[9]. Higher serum IL-33 levels in acute ischemic stroke patients correlated positively with better prognosis, as compared with those with lower IL-33 levels. These patients presented with poorer outcome[85] suggesting that IL-33 is protective to stroke.

Serotonin receptor

As earlier described, tissue Tregs express a common set of genes among various tissue Tregs, while each organ-specific Treg expresses tissue-specific genes. Unlike other tissue Tregs, brain Tregs express several unique CNS-related genes. For example, brain Tregs express serotonin receptor 7 (Htr 7), which increases cellular cAMP[86]. It has been acknowledged that cAMP promotes the Treg proliferation and thereby potentiating Treg functions[87]. Serotonin was reported to decrease Th1/Th17 cytokines, but increased Treg population in multiple sclerosis (MS) patients[88]. Serotonin further activates Tregs from the ischemic brain *in vitro* in an Htr7-dependent manner. The administration of serotonin or a selective serotonin reuptake inhibitor (SSRI) thus increases the number of brain Tregs in the chronic phase after stroke onset, improving neurological symptoms[9]. Many reports suggest that SSRI ameliorates neurological symptoms after stroke onset[89,90], although some studies did not prove that functional recovery improved[91]. It is highly likely that brain Tregs work on neuronal repair in human stroke patients.

Amphiregulin

Hypertrophic astrocytes exhibit increased Ca^{2+} signaling, which leads to the increased expression of pro-inflammatory cytokines (IL-1β, IL-6) and chemokines (CCL3, CCL5) expression, promoting the formation of glial scars[92]. Amphiregulin (Areg) is also known to suppress the production of inflammatory cytokines, including IL-6 and TNFα, in several inflammatory diseases[93].

Areg from brain Tregs has been described to suppress the excessive activation of astrocytes, so-called astrogliosis or reactive astrocytes, which is then reported to lead to a delay in the recovery from ischemic stroke or spinal cord injury[94]. Although astrogliosis would be necessary for forming scars in order to demarcate the ischemic regions from the surrounding healthy tissue, excessively activated astrocytes can produce neurotoxic factors, resulting in neural cell damage[95]. Areg further suppresses apoptosis of neurons by suppressing excessive astrocyte activation[9]. The molecular mechanism of suppression of astrocyte activation by Areg has not been completely elucidated. Among inflammatory cytokines, IL-6 is important for astrocyte activation[96]. Since Areg suppresses IL-6 expression in microglia and astrocytes *in vivo* and

in vitro[9], suppression of IL-6 by Areg may play a key role in astrocyte regulation. In addition, Areg has been postulated to be directly involved in the proliferation of neural stem cells[97].

Enkephalin

Prepro-enkephalin is produced in Regulatory T cells[98]. Pharmacologically active encephalin has been reported to be therapeutic for stroke[99]. Opioid growth factor ([Met(5)]-enkephalin) is also neuroprotective in the murine EAE model[100]. However, some studies indicate that preproenkephalin accelerates the generation of autoimmune IFN-γ-producing T cells and exacerbates EAE[101].

PPARγ

PPARγ levels are high in brain Tregs. PPARγ agonists have been reported to increase accumulation of adipose-tissue Tregs and improve insulin sensitivity[102] In particular, PPARγ agonists have a protective effect against various types of injury to the brain[103]. Thus, it is possible that PPARγ is involved in Treg expansion in the brain, which should be further investigated.

TREGS AND HUMAN CNS INFLAMMATORY DISEASES

Accumulation of Tregs in the human brain of ischemic stroke patients has not been clearly shown. However, a correlation between peripheral blood Treg/Th17 ratio or IL-17/IL-10 levels and stroke prognosis has been reported in human stroke[104-107]. An inverse correlation between the number of Tregs in the peripheral blood and the severity of stroke has also been reported in patients[13,108].

It has been established that neural inflammation plays important roles not only in cerebral infarction, but also in various types of damage to cerebrospinal tissues. These include spinal cord injury, autoimmune diseases such as multiple sclerosis, and in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Innate and adaptive immunity may be involved in these neural inflammations. Although Tregs have been shown to infiltrate and accumulate within the CNS[109] of neuroinflammatory diseases, role of Tregs in such diseases have not been well characterized. Since Tregs of MS patients have been shown to proliferate by serotonin stimulation[88], Tregs in the CNS diseases may be similar to the brain Tregs that have been characterized in a murine ischemic stroke model.

CONCLUSION

In summary, brain macrophages as well as brain Tregs play important roles in the resolution of inflammation and neural recovery. The conversion mechanism from inflammatory macrophages to tissue-repair macrophages and the mechanism of expansion of brain Tregs by recognizing self-antigens in the cervical LN and the brain remain to be described. Neuroprotective factors such as IGF-1 from macrophages and Areg produced by brain Tregs not only suppress excessive activation of microglia and astrocytes, but may also promote neural cell survival and neural stem cell recruitment [Figure 2][110,111]. The molecular mechanisms whereby macrophages and Tregs acquire brain-specific characteristics, including Maf-b/scavenger receptor expression and serotonin receptor expression, respectively, remain to be clarified. Such mechanisms could be used for increasing Tregs in the brain. Identification of brain factors and self-antigens for brain-specific macrophages and Tregs may facilitate the development of therapies for not only cerebral infarction but also other central nervous system diseases. Adoptive transfer of mesenchymal stem cells to the brain has been proposed to treat stroke patients[112]. Similarly autologous Tregs transfer into the brain is also possible for the treatment of cerebral inflammation. It is also important to define the role of brain Tregs in other neurodegenerative disorders and neuroinflammatory diseases.

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Authors' contributions

Searched literatures and wrote the manuscript: Yoshimura A, Ito M

Availability of data and materials

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Neuroimmunology and Neuroinflammation

Review

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The immune regulation of PD-1/PDL-1 axis, a potential biomarker in multiple sclerosis

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Abstract

Multiple sclerosis is an autoimmune disease characterised by a chronic inflammation within the central nervous system. In the last ten years, studies on multiple sclerosis have been concentrated on the discovery of new biomarkers of disease and potential therapeutic targets. In chronic infection or in cancer, the immune system response is faulty and maintained in a condition defined as T-cell exhaustion induced by expression of co-inhibitory receptors. The PD-1/PDL-1 pathway is demonstrated to be the main one responsible for promoting T-cell exhaustion, and immunotherapies targeting PD-1 or PDL-1 have shown beneficial clinical outcomes in several tumours and chronic diseases. Contrarily, transcriptional T-cell exhaustion signature and high expression of co-inhibitor receptor PD-1 are associated with favourable prognosis in multiple sclerosis and other autoimmune diseases. Several studies have clearly demonstrated PD-1 has a dual role in immune self-tolerance: to constrain autoreactive T cells in anergic condition and to protect the tissue from the damage caused by the activation of endogenous autoreactive T cells. Consequently, immune checkpoint inhibitor therapies that target inhibitory receptors in cancer cause an exacerbation of autoimmune diseases. This review describes the roles of the PD-1/PDL-1 pathway in cancer and autoimmune diseases, especially in multiple sclerosis, and how manipulating PD-1 can be a therapeutic approach in multiple sclerosis.

Keywords: T-cell exhaustion, inhibitory checkpoints pathways, PD-1/PDL-1 axis in autoimmune disease, multiple sclerosis, immune checkpoint inhibitor treatments, multiple sclerosis biomarkers

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T-CELL EXHAUSTION

The word "exhaustion" originates from the Latin "exaurire" and was used for the first time to explain a mechanism for silencing antiviral T-cell response during a Lymphocytic Choriomeningitis Virus infection (LCMV)[1]. Antigen-specific CD8 T cells remove viral infection by killing infected cells and the production of antiviral cytokines such as interferon gamma (IFNγ). The damping of immune response in LCMV was associated with two mechanisms silencing the CD8 Cytotoxic T Lymphocytes (CTLs) response: the depletion of nucleoprotein-specific CD8 T cells and the persistence of exhausted glycoprotein-specific CD8 T cells unable to kill virus-infected cells and release antiviral cytokines[1]. Further investigations showed that the exhaustion process suppresses the CD8 antiviral activity by the hierarchical loss of T cell function[2]. Proliferation, release of IL-2 and cytolysis were lost at an early stage of exhaustion, followed by tumor necrosis factor alpha (TNFα) production and, at the severe late stage, IFNγ production. CD4 T helper cells (Th cells) drive the fate of CD8 T-cell responses in chronic viral infections. Mice with transient depletion of CD4 T cells before infection with chronic strains of LCMV develop CD8 T-cell exhaustion and high viral load compared with non-treated mice[3]. Th cells are necessary for the generation of stable and functional CD8 memory cells[4-6]. During a chronic infection, CD8 T cells develop an exhaustion phenotype that produces a state of immunosuppression in the absence of CD4 T cells[7]. The exhaustion process induces low levels of Th cells[8-10] and affects CD4 T cell functions with loss of proliferation and IL-2 and TNFα production[11]. Moreover, CD8 T cell and B cell response was restored when functional LCMV-specific CD4 T cells were transfected in LCMV chronically infected mice. PD-1 expression increased in LCMV-specific CD4 T cells by two weeks after transfer in chronically infected mice and programmed death 1 (PD-1) blockage improved the CD4 T-cell activity[12,13]. In addition, the rescue of CD8 T cell function in terms of proliferation and cytokine release was greater in mice receiving the combination of PD-1 blockade and Th cells compared with the mice receiving either treatment alone[12].

INHIBITORY CHECKPOINT PATHWAYS

Cytotoxic T Lymphocytes A-4 (CTLA-4), PD-1 and programmed death ligand 1 (PDL-1) are the first inhibitory checkpoint receptors to be discovered and targeted in cancer immunotherapy and chronic viral infection. The amplitude of T-cell response depends on the activation of co-stimulatory (CD28) or inhibitory receptors after the engagement of T-cell receptor (TCR) with the cognate-peptide-major histocompatibility complex. Co-inhibitory receptors show distinct patterns of expression and different mechanisms of action and signalling.

The knockout CTLA-4 mice has shown a lethal hyperactivation phenotype, confirming that CTLA-4 is a vital inhibitor checkpoint of the immune system. After TCR activation, CTLA-4 upregulates in the CD4 T cells and competes with the co-stimulatory receptor CD28 for its ligands CD80 and CD86, for which CTLA-4 has more binding affinity. The link of CTLA-4 to CD80 and CD86 inhibits T-cell activation. Because antigen-presenting cells and dendritic cells express CD80 and CD86, the suppression of anti-tumour immunity by CTLA-4 is thought to occur in the secondary lymphoid organs as well as in the tumour microenvironment.

PD-1 is an inhibitory receptor that belongs to the CD28 family. The receptor has been detected on activated T lymphocytes, B lymphocytes, dendritic cells, macrophages and natural killer cells after a transcriptional activation[14]. PDL-1 is the ligand of PD-1, belongs to B7 family and is present on B lymphocytes, antigen-presenting cells (APC) and tissue cells, including several types of cancer. PD-1 engagement activates the inhibitory phosphatase PP2A and SHP-2 by immune receptor tyrosine inhibitory motif and immune receptor tyrosine switch motif, inhibits T-cell activation and increases T-cell migration within tissues.

T-cell exhaustion in cancer and infectious diseases. T-cell exhaustion has been described in animal models of polyomavirus[15] and adenovirus[16], as well as in chronic human infections mediated by human

immunodeficiency virus (HIV)[17] and hepatitis B and C virus (HBV, HCV)[18,19]. The loss of antiviral activity on CD8 T cells is associated with the upregulation of PD-1 in an animal model of LCMV followed by hierarchy suppression of cyto- and cytotoxic function[20]. The CD8 cytotoxic function against infected cells and antiviral cytokines production can be restored by blocking the PD-1/PDL-1 pathway, leading to clearance of infection in LCMV. The PD-1/PDL-1 pathway is the principal regulator of T-cell exhaustion in the animal model of LCMV. Studies on PD-1 in human chronic infections such as Human HIV and HCV have shown an increase of PD-1 on virus-specific CD8 T cells. PD-1 increases in HIV-specific CD4 and CD8 T cells and is directly correlated with the viral load and inversely with CD4 T cell counts. Furthermore, PD-1 increases in patients with HIV progression as compared with patients with long-term progression.

Moreover, T-cell exhaustion suppresses cancer immune-surveillance, leading to tumour spread. Restoring the immune surveillance by blocking PD-1/PDL-1 pathway has been an essential improvement in the cancer treatment. The function of PD-1 and its deregulations are summarised in Figure 1.

Nonetheless, some tumours develop resistance to PD-1 blocking, which is regulated by the tumour microenvironment where infiltrates of regulatory and immune suppressor cells (myeloid suppressor cells, regulatory T cells, immature dendritic cells and immune-suppressive macrophages) reduce the activity of cytotoxic CD8 T cells. Any treatment that induces changes in the levels of hormones and growth factors increases the vulnerability of cancer cells to cytotoxic drugs, which become sensitive to PD-1 treatment. Furthermore, short-term starvation (STS) has been described to reduce levels of insulin-like growth factor 1 (IGF-1) in the lung cancer microenvironment with an increase in the infiltration of immune cells and cytotoxic CD8 T cells[21]. The combining of PD-1 blockade treatment with STS boosts the immune system, reducing the tumour size significantly in a mouse model of KRAS-driven lung adenocarcinoma and Lewis lung carcinoma[21]. The combination of the two treatments induced in the mice an extended lasting memory response. The immunological study has shown an increase in tumour-infiltrating CD8 and natural killer cells by reducing the proportion of CD4 and B cells. CD8 and CD4 T cells showed a reduction in PD-1 expression. Depletion of CD8 T cells abrogated utterly the effect of the STS and anti-PD-1 treatment, confirming that STS sensitises the lung cancer to CD8 T cells reactivated by PD-1 blocking. The tumour-immune infiltrate treated with anti-PD-1 after STS was analysed with a flow cytometer and presented an increase in the frequency of tumour-specific IFN-γ-producing T cells as compared with mice treated with only one agent or vehicle[21].

A selective ablation of PD-1 on myeloid cells or T cells has essentially contributed to understanding the function of PD-1 in the cancer-immunity cycle. Mice with PD-1 ablated only on myeloid cells showed an increase of effector memory T cells and an enhanced response against the tumours. Ablation of PD-1 on myeloid cells changes the tumour microenvironment, skewing the myeloid cell fate toward differentiation of monocytes, macrophages and $CD11c^{+}MHCII^{+}$ dendritic cells (DC) rather than myeloid suppressor cells and granulocyte/macrophage progenitors. The reduction of myeloid suppressor cells due to PD-1 ablation contributes to restoring the functionality of effector memory T cells and, consequently, an immune response to the tumour[22].

PD-1/PDL-1 AXIS IN AUTOIMMUNE DISEASE

Autoimmune thyroid diseases (AIDTs) are an organ-specific autoimmune disease that affects 50/100,000 people per year, with a prevalence in females[23]. Infiltrating lymphocytes generating follicle structures are described in the thyroid glands in Hashimoto thyroiditis and Grave's disease (GD), the most common AIDTs[24]. Interferon signalling and increased expression of PD-1 and M2 macrophages markers were revealed in the transcriptomic analysis of GD glands[25]. Thyroid autoimmunity is one of the most common Immune-Related Adverse Events observed after immune checkpoint inhibitors (ICI) treatments in cancer[26].

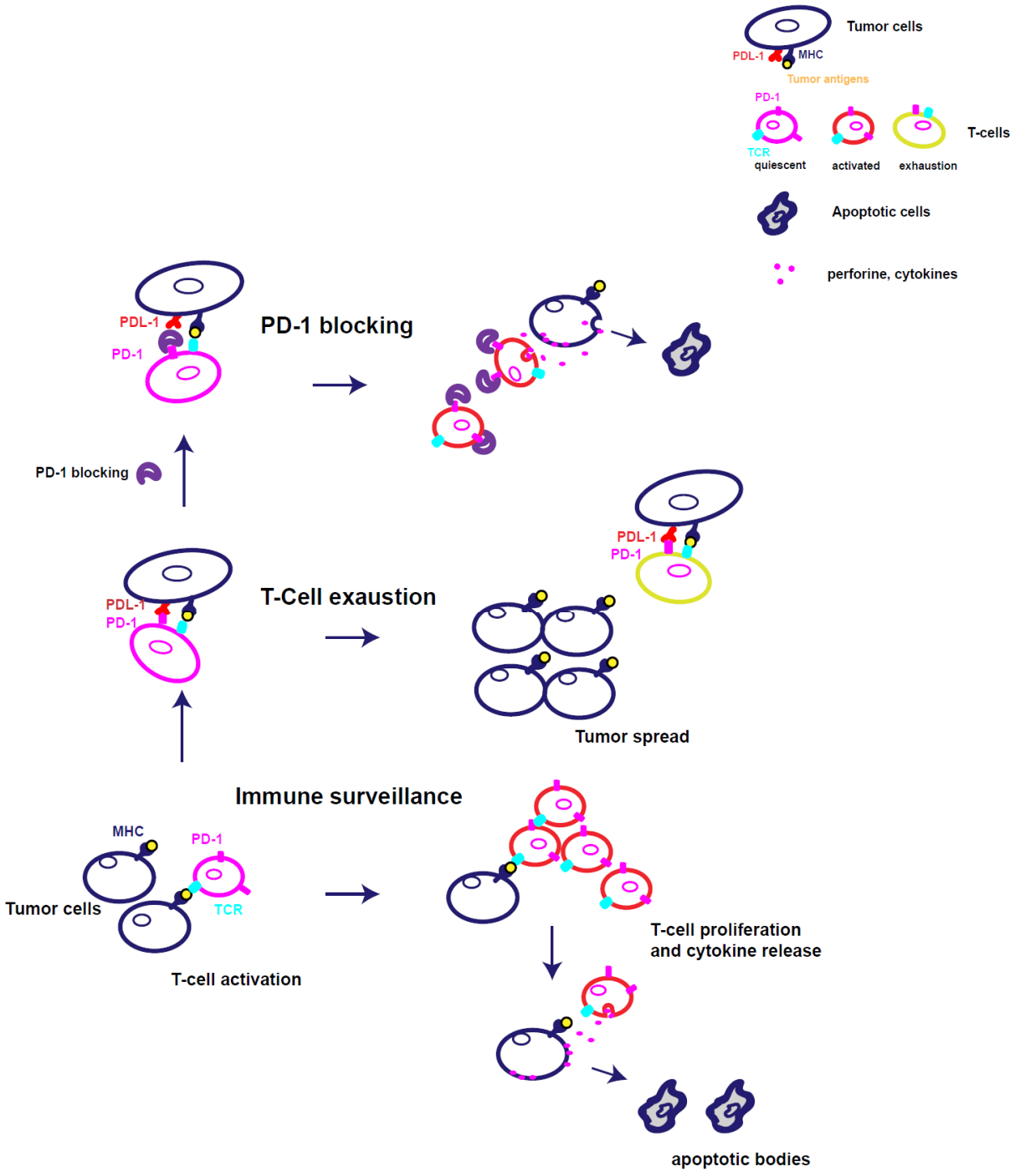

Figure 1. PD-1 inhibitor checkpoint regulates T cell activation during immune surveillance and induces T-cell exhaustion in cancer. (1) PD-1 is expressed on activated T cells and regulates the activity of late differentiate effector cells. The foreign antigens are presented to T cells by the TCR engagment with MHC expressed on dendritic cells, infected cells or tumor cells. Thus, T cells proliferate and differentiate in effector and memory cells. Effector cells kill the foreign antigens express on infected or tumor cells by releasing inflammatory cyokines and cytotoxic granules that induce target-cells to apoptosis. PD-1 is an inhibitory checkpoint able to regulate the T cell activation when the inflammation is resolved. (2) After activation, PD-1 increases on T cells and the link with the ligand PDL-1 reduces the T cell functionality. Thus, T cells are not able to kill the tumor or reduce the viral load and this condition called T-cell exaustion favours a persistent infection and tumor spread. (3) The blocking of PD-1 with monoclonal antibodies restores T cell function. T cells proliferate and differentiate in effector and memory cells contributing to resolve the infection or tumor. MHC: major histocompatibility complex

The PD-1/PDL-1 axis has been investigated in the peripheral blood and the infiltrating lymphocytes in glands of patients with GD and compared with non-multinodular goitres as non-autoimmune controls and healthy controls (HC)[27]. A decrease of naïve as well as an increase of memory and effector subsets of CD4 T cells was observed in GD as compared with the healthy donors (HD)[27]. Besides, infiltrating lymphocytes in the gland of GD patients were predominantly effector and memory cells. PD-1 was found higher in GD than HD in CD4 T cells, and it increased in effector memory T cells re-expressing CD45RA (TEMRA), effector and central memory subsets. PD-1 expression increased in infiltrating CD4 and CD8 T cells in infiltrating lymphocytes with predominance on effector and memory subsets. The expression of PDL-1 but not programmed death ligand 2 (PDL-2) was observed in epithelial thyroid follicular cells in the thyroid tissue from GD patients but not in non-multinodular goitres patients[27].

Rheumatoid arthritis (RA) is a chronic progressive inflammatory disorder characterised by damage of articular cartilage and joint destruction[28-31]. Environmental, genetic, infectious and hormonal factors can contribute to the pathogenesis of the disease[32,33]. The overproduction of TNFα generates inflammation and damages the joints. The interaction of B and T lymphocytes[34] with synovial-like fibroblasts and macrophages causes the overproduction of TNFα that induces the production of several inflammatory cytokines, such as interleukin-6 (IL-6)[35,36]. Several animal and clinical studies revealed the presence of CD4 T cells in the perivascular cuff and infiltration of CD8 T cells into the tissue. Depletion of T cells or treatment of anti-cytokines that are involved in T-cell activation or promote antigen-presentation reduces inflammation. T helper 17 cells are the primary T cell subsets involved in inflammation and autoimmunity in RA[37]. PD-1$^{-/-}$ C57BL/6 mice developed arthritis. PD-1 polymorphisms have been reported to be associated with RA[38,39]. Expression of PD-1 was detected in synovial T cells and macrophages in patients with RA[40]. In the peripheral blood of RA patients, PD-1 was significantly decreased in CD4 T cells (P = 0.002) and CD8 T cells ($P < 0.001$) as compared with HC ($P < 0.05$)[41]. DAS28 score is a measure of disease activity in RA, and PD-1 expression was found inversely correlated with DAS28 scores in RA patients[41]. Besides, CRP is an indicator of inflammation and cases with positive CRP detection had a lower proportion of PD-1^{+}CD4^{+} T cells than those with negative CRP[42].

Systemic Lupus Erythematous (SLE) is an autoimmune disease generated by the production of antibodies against self-antigens and deposition of immune complexes in different tissues. Inflammation and multisystem disorders characterise the disease[43]. The disorder affects mainly women of reproductive age with an incidence of 20-70 cases per 100,000 individuals[44,45]. Environmental, genetic and hormonal factors are relevant in the pathogenesis of the disease[46-48]. Genetic variations in the immune checkpoint genes such as PD-1, T-cell immunoglobulin domain, mucin domain (TIM) and CTLA-4 increase the susceptibility to develop the autoimmune disease as a consequence of the breakdown of immune tolerance to self-antigens[49,50]. Several single-nucleotide polymorphisms (SNPs) have been identified to affect PD-1 function and to contribute to tumours and autoimmune disease[49,50]. The frequencies of PD-1 SNPs (PD1.1, PD1.3, PD1.5 and PD1.9) were analysed in SLE patients. The PD1.5 genotype frequency was increased in Iranian, Malaysian and European patients with SLE as compared with healthy donors[51-53]. The distribution of PD1.5 C/C, PD1.5 C/T and PD1.5 T/T genotypes versus other genotypes in patients with SLE differed from healthy controls[53]. In addition, there were significant differences in the PD1.5 genotypes between patients with renal involvement and neurological involvement and between neurological involvement and HC[53]. The allelic analysis revealed that there was a significant association between PD1.5 allele frequency and SLE susceptibility[53].

Type I diabetes (TID) is caused by autoreactive cells that destroy the insulin-producing beta cells in the pancreatic islet of Langerhans[54]. PD-1 and PDL-1 protect from TID. PD-1 deficiency accelerates the onset and the frequency of TID in NOD (non-obese diabetic) mice and infiltration of T cells into the islets. PD-1 or PDL-1 but not PDL-2 blockage rapidly induces diabetes in NOD mice with an expansion

of activated glutamic acid decarboxylase (GAD)-reactive cells[55-59]. In addition, despite CTLA-4 blockage showing a negative regulation of autoimmune diabetes only in early stages of the life, the PD1-PDL-1 pathway regulated autoreactive T cells throughout the life span of the animal and appeared to be critical for progression of autoimmune diabetes[59]. Moreover, polymorphisms that reduce the function of PD-1 are associated with human TID[60]. PD-1 function was also investigated by using a model that mimics the naïve pre-immune repertoire. Fewer islet specific BDC2.5 transgenic naïve CD4 T cells were transferred into prediabetic NOD mice[61]. BDC2.5 CD4 T cells accumulated in the pancreas surrounding the islet (peri-insulitis)[61]. When BDC2.5 naïve T cells were preactivated *in vitro* and then transferred into NOD mice, the majority of them accumulated in the pancreas but within the islet (insulitis), developing severer TID[61]. The majority of BDC2.5 cells differentiate in IFNγ-producing cells. Anti-PDL-1 administration caused a conversion from peri-insulitis to destructive insulitis[61]. PD-1 on BDC2.5 naïve T cells regulate proliferation, C-X-C Motif Chemokine Receptor 3 (CXCR3) expression, infiltration of the pancreas, and release of inflammatory cytokines IFNγ, TNFα and IL-2. Moreover, PD-1 but not PDL-1 expressed by BDC2.5 cells is required to suppress proliferation and infiltration of the pancreas[61].

A fusion protein containing a single-chain variable fragment (scFv) of PD-1 antibody (aPD-1), an albumin-binding protein and *Pseudomonas aeruginosa* exotoxin A was used to select and kill PD-1$^+$ cells[62]. The treatment was tried first in animal model of TID. Depletion of PD-1$^+$ cells inhibited the development of TID in NOD mice, reducing the pancreatic infiltration of PD-1$^+$ cells as compared with the controls. Contrarily, anti-PD-1 was observed to induce a TID progression in NOD mice, suggesting that PD-1 blocking restores the proliferation and effector function of autoreactive cells. The TID progression was reduced in mice pre-treated with PD-1 depletion before PD-1 blocking, confirming that PD1 is expressed in autoreactive cells[62].

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS). Disease genetic and cellular studies sustain that autoreactive T cells are responsible for CNS damage[63,64]. Post-mortem studies showed that T and B cells infiltrate the CNS and, in the long term, develop lymphoid follicles with a functional germinal centre in the meninges and this meningeal inflammation causes white matter demyelination[65]. Further investigations demonstrated that inflammatory cytokines and molecules involved in T and B cell development and lymphoid-neogenesis increased in the cerebrospinal fluid (CSF) from post-mortem MS cases with a high level of meningeal inflammation and Gray matter demyelination, as well as in the CSF of patients with MS[66] and Gray matter damage at diagnosis[67]. Moreover, infiltration of T cells enriched the brain lesions[68] and T- and B-depleted therapies reduced activity and progression in MS[69].

IFNγ and IL-17-producing CD4 T cells have been defined as the effector populations driving CNS damage. Adoptive transfer of Th1 cells inducing experimental autoimmune encephalomyelitis (EAE) and the cytokine profile of cells isolated from the CNS of mice with acute EAE have shown that Th1 cytokines are released from infiltrating CD4$^+$ T cells and TNFα is predominantly transcribed by macrophages and microglia[70-72]. T-bet is the transcription factor regulating Th1 development and IFNγ production, and it is induced by interferon γ transducer and activator of transcription (STAT)-1 signalling pathway during T-cell activation. The role of Th1 in inducing EAE was confirmed in STAT-4 and STAT-6 deficient mice. STAT-4 pathway controls the Th1 differentiation and STAT-4$^{-/-}$ mice showed resistance to the development of AEA. Mice deficient in STAT-6, which regulates the differentiation of Th2 cells, develop severer AEA and have more Th1 phenotype[73]. The IFNγ-producing CD4$^+$T cells generate in the cervical lymph nodes and Th1 migration happens 24 h before the onset of neurological signs of EAE[74]. Although Th1 cells contribute to EAE, IFNγ knockdown mice are predisposed to develop EAE and infiltrates of lymphocytes, macrophages and granulocytes were detected in the CNS[75-77]. The results from IFNγ knockdown and STAT-1 deficient mice established the contribution of other effector cells to the disease pathogenesis.

Besides, the discovery of IL-23 rather than IL-12 being crucial for EAE development led to evaluating Th17 cells and their transcriptional factor RORgt in the EAE pathogenesis[78-80], Moreover, Th1, Th17 and Th9 were defined to induce EAE with a different disease phenotype[79,81]. In addition to effector cells in EAE[82], the mechanisms of immune regulations, including regulatory B and T cells, and expression of inhibitory receptors were investigated in EAE. CD4 T cells were observed to protect against spontaneous development of CNS autoimmunity in EAE[83], and CD4 regulatory T cells characterised by high expression of CD25 and transcription factor FOXp3 isolated from peripheral blood had a reduced effector suppression function in patients with MS as compared with healthy donors[84]. A defect in regulatory B cells was also described to induce EAE and autoimmunity in mice and patients[85,86].

Single-cell transcriptomics of blood and CSF cells isolated from patients with MS and healthy donors revealed that different mechanisms operate in the two compartments[87]. Analysis of the data showed that MS affects the cellular composition of the CSF and the transcriptional phenotype of blood cells[87]. Blood cells exhibited several transcriptional changes, including induction of activation markers (ICOS), cytokine receptors (IL17RA) and trafficking molecules (PECAM1/CD31 and ITGA5/a5 integrin) in T cells[87].

Contrarily, an enrichment of CD4 T cells with T helper 1 and T follicular helper (Tfh) profiles, regulatory T cells, myeloid lineage cells and late-stage B lineage cells were detected in the CSF[87]. Furthermore, Tfh cells expressing PD-1 were observed to correlate with the proportion of plasma cells and showed cytotoxicity and co-inhibitory function. Follicular T helper (Tfh) cells, a subset of T helper cells, are necessary for B cell differentiation and antibody production[87,88]. These cells express CXCR5, CD40 ligand and IL-21 as well as high levels of inducible T-cell constimulator (ICOS) and PD-1. They were described to migrate in the germinal centre and to activate B cells. An elevated frequency of circulating Tfh and B cells was identified in MS patients undergoing relapse and Tfh-like cells upregulated during the course of EAE progression[88]. In addition, an adoptive cell transfer experiment showed that myelin oligodendrocytes glycoprotein (MOG)-reactive Tfh-like cells induced a worsening of the disease, delaying the remission of EAE *in vivo*[88]. Despite the use of PD-1 to identify Tfh cells, the role of PD-1 signalling on Tfh cells is only beginning to be investigated. When PD-1 is engaged by its ligand PDL-1 on follicular B cells, a bystander mechanism is activated and PD-1-expressing Tfh cells are recruited into a special niche inside the GC, even though Tfh PD-1^{neg} migrates to the follicle outside of the germinal centre[89]. Moreover, both ICOS and PD-1 are requested for maintaining the stringency of affinity-based selection between Tfh cells and antigen-specific cells[89].

The transcriptional signature of CD8 T-cell exhaustion predicted better prognosis in multiple autoimmune diseases[90]. Transcriptomes of CD4 and CD8 T cells isolated from a group of patients with active autoimmune diseases were analysed to identify modules of genes with a strong correlation with relapse rate. Modules corresponding to CD4 T-cell co-stimulation were found to correlate with clinical outcomes. In detail, CD4 co-stimulatory receptors, CD2, KAT2B and other surrogate markers were described to increase in MS patients with active autoimmune disease.

The immune regulatory role of PD-1 in MS was suggested by experiments in EAE, a mouse model of MS[59,91,92]. Mice in which PD-1 was deleted or the PD-1 pathway was inhibited by blocking the link between PD-1 and its ligand PDL-1 develop a worsening EAE with an increase of infiltrating immune cells, especially CD8 T cells into the CNS[59,92,93]. The deterioration of disease in PD-1$^{-/-}$ and PDL-1$^{-/-}$ mice was related to over production of inflammatory cytokines IFNg, TNFa, IL-6 and IL-17 released by draining lymph node cells during re-stimulation *in vitro* with different concentrations of MOG[93]. PDL-1 is rarely expressed in the brains of controls[94]. Contrarily, PDL-1 was detected in the majority of lesions expressed from astrocytes and microglia/macrophages with low expression of PD-1 on infiltrating T cells in post-mortem MS brain tissues[94]. A recent publication shows that the PDL-1 in dendritic cells improves EAE in

mice. The authors used a hypomethylating agent 5-aza-2'-deoxycytidine, which reduces methylation in a "CPG" island located near the transcription site of *Cd274* gene. The reduced methylation favours the gene transcription and upregulation of PDL-1 in dendritic cells. This effect was observed in DC isolated from EAE mice pre-treated with hypomethylating agent and *in vitro* treatment of bone marrow dendritic cells with the hypomethylating agent. Furthermore, DC isolated from EAE mice pre-treated with 5-aza or bone marrow dendritic cells treated *in vitro* with 5-aza suppressed proliferation and release of inflammatory cytokines such as IL-17 and TNFα when the DC were co-cultured with CD4 T cells isolated from EAE mice. In addition, an inhibition of EAE was observed in mice pre-treated with 5-aza before EAE induction. An increase of PDL-1 and PDL-2 was detected in DC isolated from EAE mice in accordance with the *in vitro* results. Moreover, blocking of PDL-1 but not PDL-2 exacerbated the EAE symptoms, confirming that the link of PDL-1 but not PDL-2 with PD-1 is relevant in the suppression of T cell function by DC[95].

PD-1 depletion was also applied in mice immunised with a peptide of myelin oligodendrocyte glycoprotein as adjuvant to develop EAE[62]. After PD-1 depletion, the mice recovered from EAE with a clinical score of one at the end of the experiments compared with the control mice that showed a score four without recovery. Depletion of PD-1 reduced the fractions of PD-1$^+$CD4$^+$ and PD-1$^+$CD8$^+$ T cells but not B cells in the CNS as compared with the controls. Besides, PD-1 depletion did not alter the ability of the treated mice to mount an immune response. The baseline number of PD-1$^+$ cells in the blood and peripheral lymphoid organs was low, confirming that PD-1 is expressed on autoreactive cells infiltrating target organs[62].

In view of publications giving PD-1 a crucial role in protection against autoimmunity in human and animal models, Jang *et al.*[96] investigated how PD-1 controls the activation and accumulation of autoreactive T cells, by constraining them in anergic state. PD-1 is one of the checkpoint inhibitors investigated in self-tolerance and discussed previously in CD8 T cells. Self-reactive cells were deleted during thymic development[97] and, of those that survived thymic deletion, only 10%-25% preferentially differentiated into immune suppressive regulatory T cells (Tregs)[98,99]. Jiang *et al.*[96] demonstrated that PD-1 is required in culling endogenous peripheral high-affinity autoreactive CD4 T cells and protect against autoimmunity[96]. The tracking of endogenous autoreactive CD4 T cells showed that more than 90% of autoreactive CD4 T cells remained FOXp3- effectors and were not regulatory T cell precursors, despite the high TCR affinity[96]. Instead, self-reactive CD4 T cells acquired cell-intrinsic tolerance through the expression of the immune checkpoint molecule PD-1[96]. Monitoring the progeny of individual autoreactive CD4 T cell clones showed that the clones with the greatest expansion burst size and highest TCR affinity expressed high levels of PD-1 and the affinity for the self-antigen induces the expression of PD-1[96] and the absence of PD-1 converts this signal when priming with consequent cell activation. A similar mechanism was described to induce the peripheral CD8 T tolerance *in vivo*. The peripheral CD8 T tolerance is induced by resting dendritic cells and depends on activation of PD-1 and CTLA-4 pathways[100].

Several studies have analysed the gene expression and protein levels of PD-1 and PDL-1 in MS, focusing on delineating any correlation with disease susceptibility or risk of progression in MS. *PD-1* gene polymorphism has been investigated in MS, and the PD 1.3 SNP has been reported to correlate with progression of the disease, demonstrating that human polymorphisms that reduce PD-1 activity increase the risk of disease. Furthermore, a significant reduction in PD-1 expression was observed in patients with mutation as compared with donors with wild-type phenotype. Furthermore, patients bearing the mutual allele showed a lower suppression of IFNγ-producing CD4 T cells after aCD3-PD-1-microbead stimulation compared with healthy donors[101]. In addition, PD-1 and PDL-1 expression in peripheral blood mononuclear cells reduced in a cohort of patients with MS as compared with healthy donors[102]. The association of three PD-1 SNPs, namely PD-1.3, PD-1.5 and PD-1.9, with MS and disease outcome were investigated in a cohort of 203 patients with a diagnosis of relapsing-remitting and secondary-progressive MS showing any association with MS risk[103]. The expression of inhibitory receptor genes,

including *CTL-4, PD-1* and *TIM-3*, decreased in patients with MS as compared with healthy controls[104]. PD-1 is usually the most downregulated gene among the investigated inhibitors[104]. PD-1 was analysed on cytotoxic $CD8^+CD57^+$T cells in the peripheral blood of patients with relapsing–remitting MS and in T cells infiltrating the brain tissue in post-mortem MS cases. PD-1 increased in $CD8^+CD57^+$T cells in patients with stable disease and decreased in active-relapsing MS compared with healthy donors[105]. PD-1 was also found to increase in CD4 and CD8 T cells in MS patients early after autologous hematopoietic stem cell transplant[106]. A study of long-term immune reconstitution in MS patients after autologous hematopoietic stem cell transplant demonstrated that an early expansion of $CD8^+PD\text{-}1^+$T cells and $CD19^+PD\text{-}1^+$B cells is associated with favourable neurological outcomes[106]. PDL-1 was also investigated in post-mortem MS brain tissue. In MS lesions, glial cells with elevated PDL-1 and PD-1 expression were found absent in many infiltrating CD8 T cells[94]. Moreover, PDL-2 but not PDL-1 is expressed in human brain endothelial cells under basal culture conditions whilst both are upregulated under inflammatory condition[107]. PDL-1 or PDL-2 blockade lessens CD8 and CD4 T cell transmigration and CD8 T cells response[94]. Furthermore, PDL-1 is undetectable in the brain endothelium in normal tissues and MS lesions, even though PDL-2 is detectable in all blood vessels in normal brain tissue and in 50% of MS lesions[94].

MULTIPLE SCLEROSIS DIAGNOSIS AFTER CANCER IMMUNOTHERAPY

ICI treatments are immunotherapies engaged in restoring the immune response to tumour or viral infection by blocking the inhibitory pathways mediated by CTLA-4 and PD-1. ICI treatments have induced neurological immune-related adverse events. Patients with an MS history developed relapses after ICI treatment for melanoma[108,109] and a biopsy of the lesions revealed acute/inflammatory demyelination without any evidence of tumour cells[109]. A comparative functional profiling of myelin-reactive T cells of patients after ICI- treatment and 14 age/sex-matched patients with MS and healthy controls was performed. Myelin-reactive T cells isolated from ipilimumab-treated patients and MS patients showed a similar autoimmune response to myelin antigen but distinct from healthy controls. That confirmed that ICI treatment causes a reactivation of self-antigen cells in MS patients. The lack of outcomes to the ICI treatment in tumour is associated with an effect on the neurological condition. A case reported maintaining stable MS during ipilimumab treatment for melanoma that did not respond to the therapy, and the patient died from metastatic melanoma[110].

Furthermore, a 29-year-old man with metastatic melanoma underwent two cycles of ipilimumab before developing MS. The TCR repertoires of tumour-infiltrating T cells isolated from the primary melanoma and those of T cells isolated in two CSF samples, five and thirteen months after the second course of ipilimumab therapy, were analysed and compared. Distinct clonotypes of CD4 and CD8 T cells in the melanoma and the CSF were identified, demonstrating that the protective antitumor response and the anti-CNS response target different antigens[111]. Outcomes of MS relapse after ICI treatment were reported in a meta-analysis study including the published literature, the analysis of food and the drug administration adverse event reporting system database and a detailed case[112]. Fourteen cases were identified with MS, of which eight had a reported history of MS. All patients presented rapid disease progression, and two of them died from severe MS after ICI treatment[112]. The median age of MS diagnosis was 52.5 years, and ICI treatment was used as immunotherapy in several types of cancer: melanoma, non-small cell lung carcinoma, pleural mesothelioma, renal cell carcinoma and colorectal cancer[112]. ICI treatments such as nivolumab, ipilimumab, pembrolizumab and atezolizumab have caused MS relapse. In addition, Isitan and Wesley[113] described the case of a 49-year-old woman with a history of relapsing–remitting MS reported to develop a severe progressive MS after atezolizumab (monoclonal antibody targeting PD-L1) therapy for metastatic colonic adenocarcinoma. The women died after her first dose of atezolizumab. Although ICI therapy has given beneficial outcomes in cancer and infectious diseases, this treatment has shown neurological side effects in patients with MS history, inducing a rapid worsening of neurological conditions. The examined cases showed a worsening of the conditions associated with the activation of T

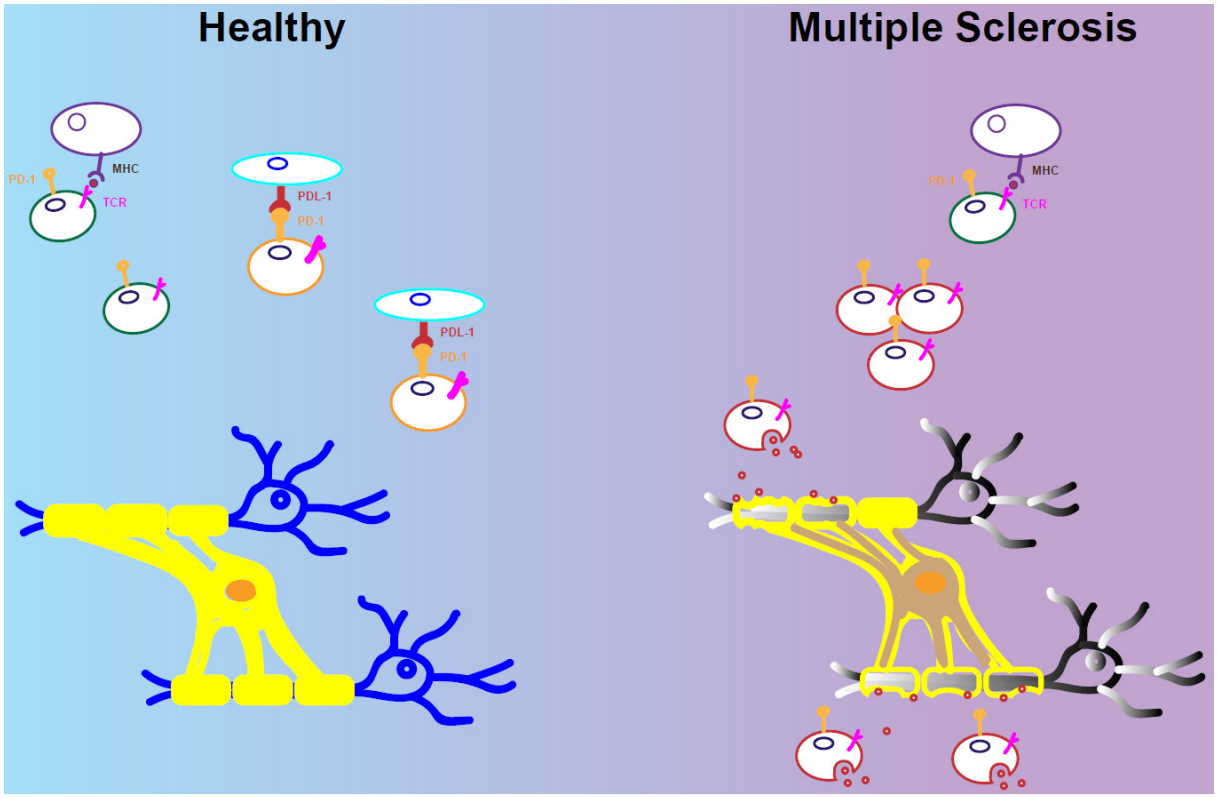

Figure 2. Immune checkpoints inhibitor treatment induces reactivation of multiple Sclerosis. PD-1 has been described on autoreactive T cells and TFH cells in multiple sclerosis. The link of PD-1 with the ligand PDL-1 controls T cell activation mantaining tollerance (Healthy). The blocking of PD-1 breaks the tollerance by reactivation of antigen-specific cells and causing damage of the target-cells with persistent inflammation in the CNS (Multiple Sclerosis). Contrary, depletion of PD-1$^+$ T cells eliminates activated antigen-specific cells reducing the T cell infiltrates and inflammation in the target-organ (PD-1 blocking). TFH: T Follicular helper cells

cells recognising self-antigen in the CNS. The relevance of PD-1 blocking and depletion of PD-1$^+$ cells in the pathogenesis of multiple sclerosis is shown in Figure 2.

CONCLUSION

PD-1 is an immune checkpoint inhibitor demonstrated to reduce the immune system response in cancer and chronic infection. Recent investigations have highlighted the dual role of PD-1 in immune tolerance, and the loss of PD-1 causes autoimmune diseases. Depletion of PD-1$^+$ T cells has given beneficial effect in autoimmune disease, slowing down the inflammation and disease progression. A decrease of PD-1 is predisposed to autoimmunity, as described in experiments of PD-1 blocking or knockout in mice. PD-1 could be a target of immunotherapies in MS, although further investigations are required to define the role of PD-1 in MS. The majority of information has been derived from animal models and sporadic studies in humans. To this purpose, expression and levels of PD-1 and PDL-1 in the peripheral blood, CSF and post-mortem MS brain tissues and the correlation of their levels with risk of MS disease and inflammation could make relevant contributions for considering PD-1 a target in MS immunotherapies.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

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Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Neuroimmunology and Neuroinflammation

Review

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What is the role of Brain derived neurotrophic factor in Multiple Sclerosis neuroinflammation?

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Abstract

Multiple Sclerosis (MS) is a chronic, inflammatory and degenerative disease of the central nervous system (CNS) with an unknown etiology. The MS pathophysiology is due to altered bidirectional interactions between several immune cell types in the periphery (such as T and B cells, myeloid cells) and resident CNS cells (such as microglia and astrocytes). It is also known that inflammatory responses have both detrimental and neuroprotective effects. The release of brain derived neurotrophic factor (BDNF) by immune cells, in both peripheral blood and into inflammatory lesions in MS, but also by microglia and astrocytes, into the CNS, seems to be a possible mechanism for this neuroprotective effect. So far, the link between BDNF and neuroinflammation has been poorly investigated. A better understanding of this link could help in the development of new therapeutic strategies for MS. In this review, the role of BDNF in MS will be discussed as well as its possible alternative as an innovative therapeutic target.

Keywords: Multiple sclerosis, neuroinflammation, brain derived neurotrophic factor, neuroprotection, neurotrophin, therapeutic target

INTRODUCTION

Multiple Sclerosis (MS) is a chronic, inflammatory and degenerative disease of the central nervous system (CNS)[1,2] of which the etiology is unknown. The clinical course of MS is characterized by fluctuating

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neurological symptoms in most patients, with early clinical relapsing-remitting episodes and/or radiological worsening and different degrees of recovery (RRMS)[3]. The relapsing-remitting phase, in most patients, is subsequently followed by a chronic progressive phase; approximately 10%-15% of patients show a progressive form of the disease from onset.

For some authors, MS is an exclusive autoimmune inflammatory disease caused by dysregulated auto-reactive immune cells that traverse the blood-brain barrier (BBB) into the CNS parenchyma, attacking various cell types (the "outside-in" autoimmune hypothesis). For other authors it is a primary degenerative disease (the "inside-out" hypothesis) in which inflammation is secondary to the release of auto-antigens (components of myelin oligodendrocyte glycoprotein, myelin basic protein, and proteolipid protein), promoting autoimmunity[4]. Thus far, it is difficult to discern whether the inflammatory processes of MS are a product or a cause for neurodegeneration with a background autoimmune etiology. In all phases of the disease both immune and degenerative processes appear to coexist and this makes it difficult to definitively resolve the "outside-in" *vs*. "inside-out" controversy.

Despite this, there is well-documented evidence that, in MS, an uncontrolled inflammatory response in the CNS (neuroinflammation) causes destruction through high levels of pro-inflammatory cytokines, proteases, glutamate, and free radicals. Consequently, immunomodulatory drugs that reduce or suppress the activity of immune cells have been successfully used to reduce clinical relapses in MS and/or neuroradiological "activity", which are associated with the entry of leukocytes through the BBB[5]. Sustained disability, however, is due to a progressive neurodegenerative process, ending with axonal loss and brain atrophy, primary or secondary to the peripheral and compartmentalized inflammation in the CNS[6]. To date, no approved therapy has provided marked neuroprotective effects nor have commonly anti-inflammatory therapies, used in the treatment of the disease, showed great efficacy in the progressive phase of MS.

Neuroinflammation have not only harmful but also neuroprotective effects[7,8]. In MS and other neurological diseases, the reparative activities of inflammatory response have been demonstrated[9]. Therefore, some authors introduced the concept of "neuroprotective autoimmunity"[10,11]. The release of neurotrophins by immune cells in both peripheral blood and directly into inflammatory lesions in MS[12,13], but also by microglia and astrocytes in the CNS, stimulating neuronal growth and survival, seems to be a possible mechanism for this neuroprotective effect[14]. Among neurotrophins, brain derived neurotrophic factor (BDNF) seems to be a good candidate in promoting the beneficial effects of inflammation in MS.

In this review, the role of BDNF in MS neuroinflammation and as a novel therapeutic target will be discussed.

NEUROINFLAMMATION: THE DETRIMENTAL EFFECT

MS pathophysiology is characterized by altered bidirectional interactions between several immune cell types in the periphery and resident cells of the CNS, such as microglia and astrocytes[15]. The MS relapses, typical in the early phases of disease, are characterized by the infiltration of pro inflammatory CNS-specific effector T cells (CD4+ and CD8+ T cells), B cells and myeloid cells into the CNS parenchyma, that are activated and/or regulated in an aberrantly way[16]. The altered function of regulatory T (Treg) cells and resistance of CNS-specific effector T cells to Treg cell-mediated regulation could be a possible cause of the neuroinflammation[17-21]. Furthermore, CNS-resident cells, that secrete many inflammatory mediators, recruit inflammatory cells into the CNS. Microglia and astrocytes in particular, can also produce cytokines, chemokines and reactive oxygen species in the presence of homeostatic disturbance, promoting and sustaining axonal damage and neurodegeneration in MS[16]. Therefore, both peripheral and CNS-compartmentalized inflammatory mechanisms contribute to MS pathogenesis[22].

In the advanced stages of the disease, the infiltration of immune cells into the CNS is reduced, whereas ongoing CNS-compartmentalized inflammation seems to dominate progressive phases of MS. During this progressive phase, the role of B cells in driving inflammation seems to be prominent, particularly within meningeal inflammation[23]. In this phase, the B cell functions are antibody production, cytokine secretion, antigen presentation and ectopic formation of follicle-like structures[23-25]. The latter seem to maintain a high level of humoral response and other autoimmune mechanisms, in the CNS, independently from peripheral inflammation. This is very relevant during progressive MS phase, with the BBB being relatively intact and the contribution to disease from entry of peripheral immune cells into the brain fairly exiguous[26].

Activated microglia also plays a central role in neuroinflammation because it can sustain ongoing inflammation[27]. Microglia activation, in MS, is diffusely present in the lesions, in normal-appearing white and in grey matter[28]. Activated microglia, secreting pro-inflammatory cytokines, such as IL-1, IL-6, TNF-alfa, and IFN, promoting phagocytic activity, and presenting antigens via MHC Class II to CD4+ T cells[27], causes damage to oligodendrocytes; moreover, microglia inducing mitochondrial dysfunction, through reactive oxygen and nitrogen species, contributes to neuronal damage[29].

In addition, activation of astrocytes into demyelinating lesions contribute to oligodendrocyte injury and axonal degeneration[30]. So far, theories of either innate immune cells in the CNS are dysregulated and drive primary degeneration, or react against an unidentified primary injury causing tissue damage remain unknown.

NEUROINFLAMMATION IN MS: THE NEUROPROTECTIVE EFFECT

Evidence for neuroprotective functions of immune cells

Some evidences have unexpectedly shown that some cells of the immune system might have a protective function during inflammation. This neuroprotective effect may be partially mediated with the production of anti-inflammatory cytokines (TGF-beta, IL-10 *etc.*) and secretion of pro-inflammatory cytokines (such as IL-6, IFN-gamma, TNF-alfa) in a dose- and time-dependent manner[31-33]. Immune cells also induce neuroprotection by production and local secretion of neurotrophic factors[11,12,34]. neural growth factor (NGF) was the first neurotrophin shown to be produced by T and B lymphocytes, macrophages, and mastcells[35]. The expression of BDNF by immune cells was also subsequently described[12]. In particular, CD4+ and CD8+ T lymphocytes, B lymphocytes, and monocytes in the human peripheral immune system can produce BDNF[12]. Moreover, neurotrophin receptors expressed by immune cells can also be targeted by autocrine or paracrine neurotrophin actions. Therefore, neurotrophins seem to mediate bidirectional cross-talk between the immune and nervous systems[11].

Evidence for neuroprotective function of microglia and astrocytes

Resident CNS cells also exercise a defensive action against immune-mediated attacks, aside from being involved in neuroinflammation. Microglia has an important role in neuroprotection and this action seems to be time-dependent. Acutely activated microglia produces inflammatory mediators that recruit other activated immune cells, amplifying the inflammatory damage, but chronically activated microglia may have a neuroprotective effect supporting the growth and survival of neural progenitor stem cells[31,36]. On oligodendrocyte precursor cells microglia seems to have always direct protective action, being the detrimental action mediated by astrocytes[37].

The neuroprotective function of microglia is mediated by different mechanisms such as debris clearance, production of growth factors (overall BDNF), production of the immunosuppressive cytokine IL-10 and neuronal circuit-shaping[27,38].

TGF-beta secretion and CTLA-4 expression produced by neurons induce CD4+CD25-effector T cells to take regulatory phenotype that exerts bystander suppression in experimental autoimmune

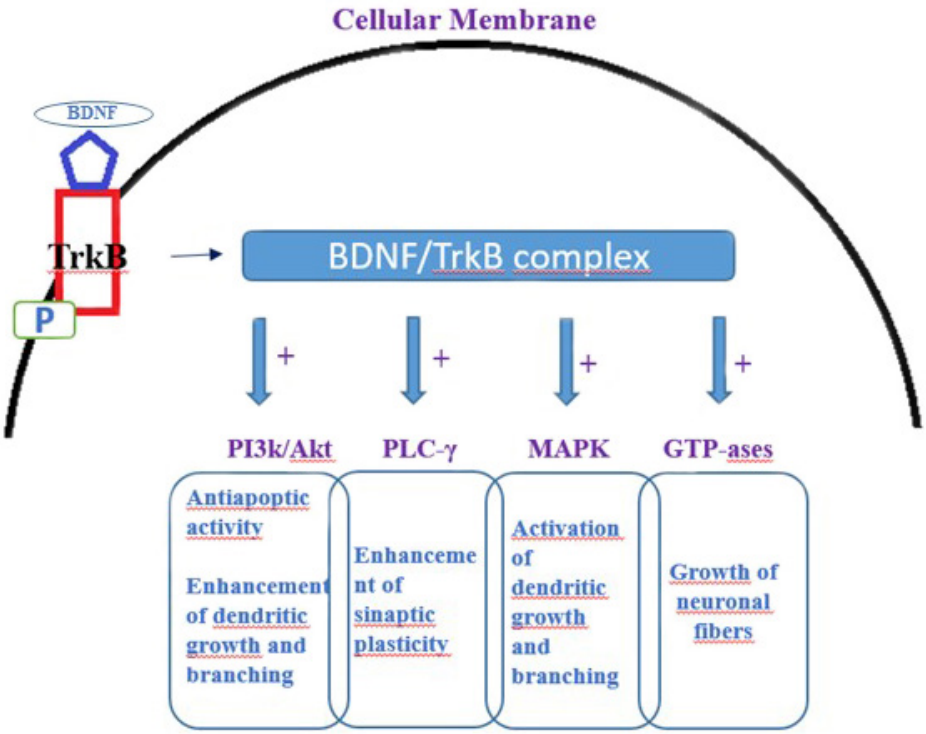

Figure 1. Intracellular signaling cascades induced by interaction of mature (m-)BDNF with TrkB receptor. Binding of BDNF to TrkB receptor induces its phosphorylation and translocation to cellular membrane. The BDNF/TrkB receptor complex triggers signaling pathways mediated by activation of PI3K, MAPK, PLC-γ, and GTP-ases. All these pathways induced by BDNF cause the enhancement/activation of dendritic growth and branching and growth of neuronal fibers. TrkB: tyrosine kinase B; BDNF: brain derived neurotrophic factor; PI3K: phosphoinositide 3-kinase; Akt: Protein kinase B; PLC: phospholipase C; MAPK: mitogen-activated protein kinase; TrkB: tropomyosin receptor kinase B

encephalomyelitis (EAE)[39]. Self-associated molecular patterns expressed by resident neurons and astrocytes drive innate cell immune responses toward a less inflammatory response[40]. Astrocytes also cause apoptosis of activated immune cells and drive microglial activity towards a less inflammatory pattern[41,42].

THE ROLE OF BDNF IN MS NEUROINFLAMMATION

BDNF

BDNF is a member of the neurotrophins gene family that includes also NGF and neurotrophins 3 and 4 (NT3 and NT4)[43] and is the neurotrophin most expressed in the brain by numerous cell types[44,45]. It plays a critical role on neuronal and oligodendroglial growth and survival, in healthy brains and in several neurologic diseases[46]. Interestingly, BDNF also modulates inflammatory homeostasis in the injured CNS[47,48].

The BDNF gene consist of a common 3′-exon that encodes the pro-BDNF region of the protein, and several species-dependent 5′-noncoding, promoter-regulated regions, terminating in a coding 5′-exon that contain the gene expression[49,50]. BDNF is translated as a proneurotrophin (pro-BDNF) that can be cut in the mature form. Both mature BDNF and pro-BDNF bind to the low affinity p75 neurotrophin receptor, activating the apoptosis cascade[51,52]. Mature BDNF binds to its high-affinity receptor tyrosine kinase B (TrkB), activating several signalling cascades[53,54] [Figure 1]. Among these, an increase in Ca^{2+} intake, phosphorylation of transcription factors, and *de novo* expression of the *BDNF* gene can be induced[53]. The nuclear factor-kappa B (NF-κB), a transcription factor with the ability to increase the expression of several pro- and antiapoptotic genes, including BDNF, is one of the main factors of inflammatory activation[55]. The

binding of BDNF to the TrkB receptor can also induce the expression of NF-κB but, the pathways for this modulation are not yet completely understood[54]. Thus, the role of BDNF in neuroinflammation, is strongly linked to its ability to induce, and being induced by, NF-κB.

Lai *et al.*[56] recently demonstrated that BDNF modulates inflammatory homeostasis, reducing inflammatory activity on microglia also through the erythropoietin and sonic hedgehog signalling pathways. BDNF may also influence the microglia inflammatory response differently in male and females probably by driving activated microglia responses toward a less inflammatory pattern in females[57].

The understanding of BDNF function in humans has greatly benefited from the identification of an SNP in the *BDNF* gene that causes a valine (Val) to methionine (Met) substitution at codon 66 (Val66Met, c.196G>A, dbSNP: rs6265). In this Met variant form of BDNF carriers, that is, BDNF Val/Met heterozygotes and Met/Met homozygotes, the pro-domain structure of the gene is altered[58]. The polymorphism can potentially alter BDNF protein-protein interactions, binding affinities, localisation, or conformational stability of the protein. Whether the polymorphism has any significant impact on the proteome profile or posttranslational modifications of various proteins in the neuronal tissues or body fluids is currently unknown[58]. Several studies have emerged implicating the association or otherwise of this polymorphism with MS. So far, no conclusive data have been published[59]. New advances in the epigenetic field, highlight the role of BDNF antisense RNA (BDNF-AS), a naturally conserved long noncoding RNA, and of DNA methylation, in the regulation of BDNF expression in MS and in several neurological diseases[60-63]. So far, only few studies have been published on this argument[64].

BDNF in MS

BDNF is the neurotrophin which is expressed more in inflammatory brain lesions of MS patients[12,13]. A significant amount of BDNF was found in infiltrating immune cells, overall in T cells and macrophages, and in neurons and astrocytes[11]. BDNF is expressed by immune cells in actively demyelinating areas of MS lesions but not in lesions without ongoing myelin breakdown. Moreover, the neurotrophin is expressed more in the actively demyelinating edge of the plaque in the early phase of its development. It is released near to axons, not directly attacked by activated immune system cells but is at high risk of bystander damage[13]. Outside MS lesions, neurons are the major source of BDNF[13]. The literature data agree in showing that neurons are the major targets for neurotrophic interactions in the CNS. In particular, the full-length isoforms of TrkB (receptor for BDNF and NT4/5) and TrkC (receptor for NT3) are usually expressed on neuronal cells. Neurons close to MS plaques showed a prominent expression of full-length TrkB (gp145TrkB)[11]. Moreover, TrkB is upregulated in a part of damaged neurons. It is known that BDNF can be anterogradely transported and released by neurons. This process is up-regulated after axonal injury and transection[65]. The common occurrence of axonal damage in MS suggests that neuronal BDNF might contribute to endogenous neurotrophic support in MS plaques[66,67].

In older and chronic MS plaques, endogenous neurotrophins are low[13]. This may be one cause for the ongoing axonal degeneration in the chronic progressive stage of MS[68-70].

In the relapsing phase, levels of BDNF are generally reported to be increased in peripheral blood mononuclear cells (PBMC) and serum[71,72], but Azoulay *et al.*[73] found less BDNF in the serum of RRMS patients with no difference in remission and relapse phases. In MS patients, serum and CSF levels and PBMC secretion of BDNF are reduced compared to healthy controls[74,75]. In line with neuropathological findings[68-70], BDNF production by immune cells in RRMS patients is higher compared to progressive MS, suggesting again that progression of MS may be due to a failure of neuroprotection and neurorepair functions under chronic injury[72,75].

Numerous studies tried to correlate the role of rs6265 BDNF polymorphism with the prognosis of MS patients, with conflicting data so that other mechanisms could be involved in the modulation of BDNF gene[59]. Latest advances in the field of epigenetics highlight the role of epigenetic mechanisms, such as methylation, in controlling key biological processes. The presence of rs6265 SNP is not a prognostic factor for reaching a more severe Expanded Disability Status Scale (EDSS)[64]. When the percentage of methylation of the *BDNF* gene is considered, a lower percentage is associated with higher odds in achieving significant disability regardless of its polymorphism. Being a higher methylation a "silencer" of the gene, a lower inhibition of the gene correlates with a high probability in achieving an EDSS score of 6.0. Patients with more severe inflammation could appeal to a de-methylation to have a higher secretion of BDNF, preserving better CNS functions. The same patients tend to reach a more severe disability score by depleting the functional reserves of the brain at a faster rate[64]. If BDNF methylation is considered as an epiphenomenon of the disease activity (or better of the neuroinflammation status), it might help to differentiate patients with a higher degree of inflammation from patients with a lower ones. If these data will be confirmed by other studies, BDNF rs6265 polymorphism methylation could become a valid prognostic factor in MS to precociously recognise patients with a more severe disease from those with a milder one[64].

BDNF AS PROMISING THERAPY IN MS

MS, but also many other CNS diseases, are tricky to treat due to the difficulty of drugs to cross the BBB. To do this, a drug must have the appropriate physicochemical properties. Alternatively, some drugs may be directly injected into the CNS but these invasive procedures are not risk-free.

Most available MS treatment have an exclusive anti-inflammatory effect helpful in reducing clinical and neuroradiological relapses but ineffective in preventing axonal loss and neurodegeneration. On the other hand, neuroprotective and/or remyelinating molecules failed to achieve the primary endpoint in clinical trials[76,77]. Conversely, brain delivery of BDNF has a potential role in reversing neurodegenerative diseases[78,79] but, so far, not through systemic administration. Therefore, there is an urgent need for development of a non-invasive trans-BBB delivery method. All the therapeutic strategies designed for delivery of neurotrophins are well summarised in the review by Huang and Dreyfus[80].

Recently, the possibility to deliver BDNF in a non-invasive way into the CNS through a BBB modulator, the ADTC5, has been found[81]. BDNF + ADTC5 delivered to the brains of mice with RR-EAE via systemic administration, significantly improve the clinical body scores of EAE mice and induce remyelination, compared to controls. Further studies are needed to confirm these data and to definitively find the best way to delivery BDNF in CNS via systemic administration.

CONCLUSION

Few studies investigated the link between BDNF and neuroinflammation even if, in many brain disorders, neuroinflammation and altered BDNF expression are commonly found. Better understanding of the interaction between BDNF and neuroinflammation could help in improving the knowledge of diseases pathogenesis and in developing of new therapeutic strategies for CNS disorders.

In MS, a large body of neuropathological, experimental and clinical evidences shows that BDNF may play an important role in neuroinflammation modulation, neuroprotection and neurorepair. These data make BDNF a good candidate for new therapeutic strategies in MS. But, when growth factors are considered as possible treatments in brain disease, some issues have to be taken into account: first, how to increase growth factors levels within specific regions of the CNS; second, how to optimize entry of growth factors from the periphery; third, to define the rate at which BDNF is taken up by the brain; fourth, the need to better understand the pharmacological characteristics of BDNF-based substances. Further studies are needed to define these aspects.

However, the challenge for neuroprotection in MS is greater than in other brain disease, because MS requires the association of both neuroprotective and immunomodulation therapies. Any exclusive inflammatory suppression is likely required to abolish both destructive and protective components and any neuroprotective treatment cannot work without a powerful anti-inflammatory therapy.

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Authors' contributions

The author contributed solely to the article.

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The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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