Mediators of Inflammation

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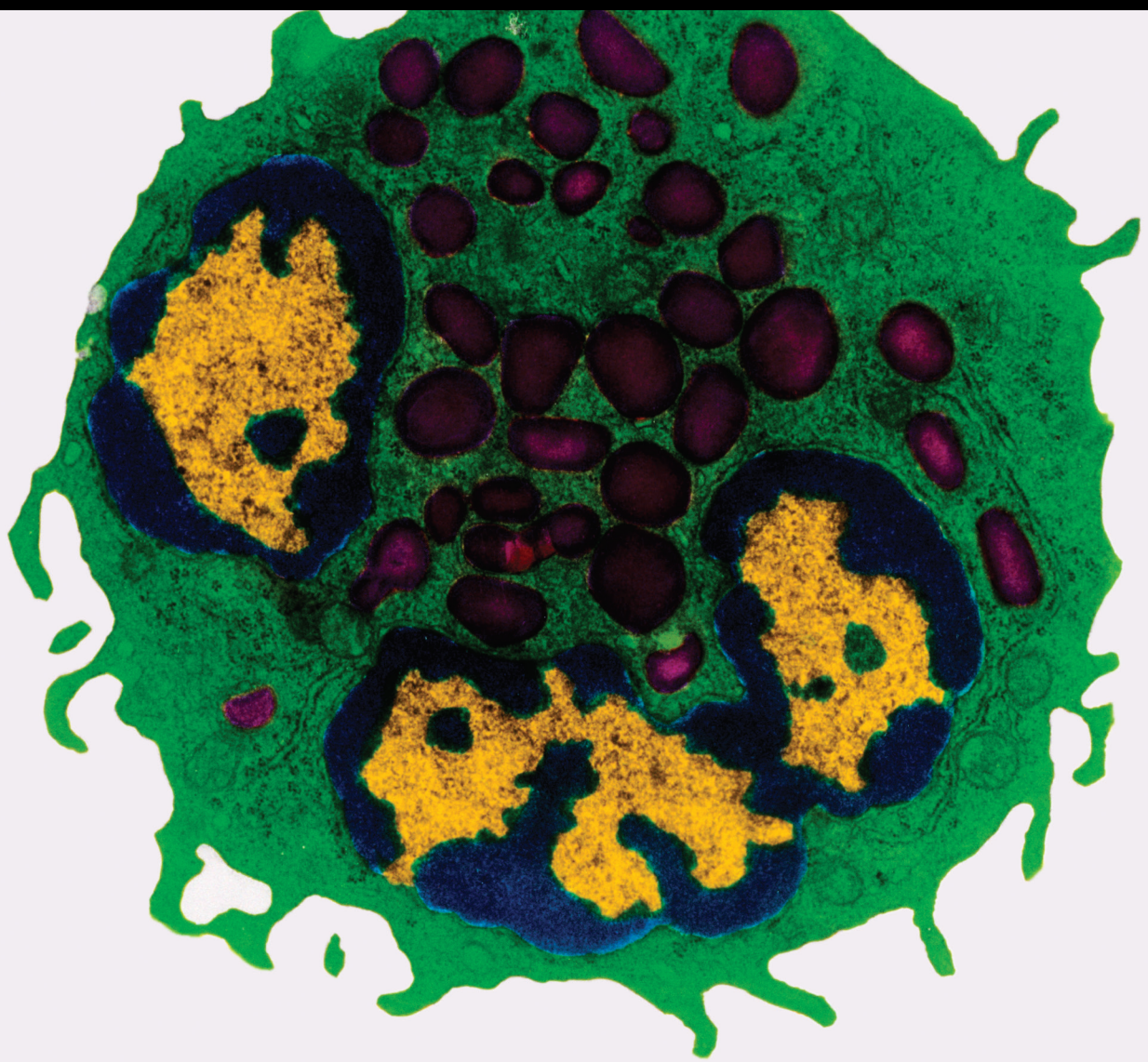

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Review Article

Microglia in Alzheimer's Disease: A Favorable Cellular Target to Ameliorate Alzheimer's Pathogenesis

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Microglial cells serve as molecular sensors of the brain that play a role in physiological and pathological conditions. Under normal physiology, microglia are primarily responsible for regulating central nervous system homeostasis through the phagocytic clearance of redundant protein aggregates, apoptotic cells, damaged neurons, and synapses. Furthermore, microglial cells can promote and mitigate amyloid β phagocytosis and tau phosphorylation. Dysregulation of the microglial programming alters cellular morphology, molecular signaling, and secretory inflammatory molecules that contribute to various neurodegenerative disorders especially Alzheimer's disease (AD). Furthermore, microglia are considered primary sources of inflammatory molecules and can induce or regulate a broad spectrum of cellular responses. Interestingly, in AD, microglia play a double-edged role in disease progression; for instance, the detrimental microglial effects increase in AD while microglial beneficiary mechanisms are jeopardized. Depending on the disease stages, microglial cells are expressed differently, which may open new avenues for AD therapy. However, the disease-related role of microglial cells and their receptors in the AD brain remain unclear. Therefore, this review represents the role of microglial cells and their involvement in AD pathogenesis.

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder that is well characterized by complex cellular and molecular alterations, such as loss of neurons and synapses, protuberant gliosis, dystrophic neuritis, formation of extracellular deposits of amyloid β (Aβ), and intracellular aggregated phosphorylated tau [1, 2]. Interestingly, reactive gliosis includes changes in function and morphology of astrocytes and microglia [3–5]. The neuroinflammatory process plays an important role in several neurological diseases, including autoimmune ailments [6]. In the case of AD development, the inflammatory response has been undoubtedly connected. Moreover, microglia have been found to have a pivotal role

in the pathogenesis of sporadic AD [7–9]. M1 microglia produce inflammatory mediators, which cause inflammation and neurotoxicity, while M2 microglia produce anti-inflammatory mediators, resulting in anti-inflammatory and neuroprotective effects. Microglia-facilitated neuroinflammation is a dual-edged sword in neurodegenerative events, with both damaging and beneficial consequences [10].

Activated microglial cells surround the Aβ plaques during Aβ phagocytosis/compaction, which may play either a neuroprotective or neurodegenerative role that depends on microglial phenotype switching [11–14]. In fact, via a reduction in the levels of Aβ in amyloid precursor protein- (APP-) based models, chronic microglial activation might improve the AD pathology [15]. Nevertheless, it has been implicated that inflammatory response exerts harmful neurotoxic effects via the release of neurotoxins and proinflammatory chemokines/cytokines [16, 17]. Induction of inflammation is also likely associated with tau pathology [18]. Evidence suggests that microglia have been linked to tau pathology and spatial memory deficits [19].

Human genome-wide association studies (GWAS) further strengthened the relationship between microglia and AD pathology. GWAS data showed that the microglial immune response is associated with multiple polymorphisms [8, 20, 21]. On the other hand, within a diverse range of AD-related genes, the microglial triggering receptor expressed in the myeloid cell 2 (*TREM2*) gene appears to have a critical contribution in case of AD-related immune response [8]. TREM2 is a lipoprotein sensor and lipid that encourages reactive microgliosis via its DNAX activation protein of 12 kDa (DAP12, a transmembrane protein) [22, 23]. It has been exhibited that through its interaction with apolipoprotein E (APOE), TREM2 controls the transcriptional activation of microglial cells [24–26]. Nonetheless, the impact of TREM2-facilitated microglial activation in AD pathogenesis, or the activities of the microglial cell, is not yet well-explained [27].

In the case of AD individuals, the neuroinflammatory response is possibly not entirely beneficial or harmful. Indeed, an uncontrolled microglial reaction might be detrimental to the surrounding neuronal elements or neurons [28]. In AD mouse models, parabiosis experiments revealed that, with a negligible contribution of infiltrating macrophages, microglia are responsible for increasing the number of myeloid cells observed in brains with plaque pathology [29]. Furthermore, via the elimination of undesirable synapses and neurons (i.e., immature synaptic connectivity whereby less active synaptic connections are formed), microglia also contribute to the developmental sculpting of neural circuits [30, 31]. These microglial roles have been shown to be compromised in aging that contributes to AD progression [32–34]. Therefore, this review is aimed at discussing how microglia act as immune system cells and how this system is changed in AD pathogenesis.

2. Microglia in Brain Aging

Aging causes microglial morphology changes [35, 36]. It has been specified in mice that microglia surveying processes are not so dynamic and less critical because of age [32, 37]. This explains the impact of pathogenic response, response to accumulated protein, or delayed injury in aged brains rather than younger mouse brains. In a facial nerve axotomy study, microglial proliferation during aging remained significantly higher in response to neuronal injury, suggesting that regulation of microglial proliferation changes with aging [38]. Moreover, the migration rate of the microglial cell was affected by aging when microglia responded to injury [32, 39]. A study on the dynamic behavior and morphology of microglia with aging disclosed that microglial response significantly reduced with age [39], whereas the distal branches become thinner and contain major functions [40–42]. Most importantly, myelin fragmentation has a role in the formation of myelin inclusions [43]. In addition to this, aging can cause the reduction of the somatic volume of the microglial cell that reduces tissue distribution homogeneity [44, 45].

During aging, microglia show an increased inflammatory response and exhibit differential changes in expression level [35]. For example, the expression level of major histocompatibility complex (MHC) II and cluster of differentiation (CD) 68 was higher in the aged microglial cell [46, 47]. On the other hand, the CD200 shows a decreased expression [48]. In order to form the ramified microglia, the CX3CL/fractalkine cytokine also plays a similar role. CX3CL1 connects with C-X3-C motif chemokine receptor 1 (CX3CR1), which is expressed vastly in the microglial cell [49]. Generally, in the pathway of canonical signaling for transforming growth factor-beta (TGFβ), Smad3 takes part in signaling, and the aged brain cell shows reduced anti-inflammatory functions [50]. Moreover, in proinflammatory gene transcription, interferon-gamma (IFNγ) activates microglia [51, 52], and the activation increases in the aged brain. Microglial maturation is influenced by altering gene function, which is predicted as a principal regulator of aging-associated changes in the microglial cell [53]. Apart from this, Iba-1 is highly expressed in microglia, which exerts its lessened ramified structure of microglial cells during aging [54], and it also accomplishes the proliferation of microglia.

According to the previous literature, microglial age-related phenotypes vary based on central nervous system (CNS) compartments [55]. The current studies have also reported that the aging effects on the microglial transcriptome are predominantly reliable on the basis of CNS locations [56]. Normally, microglia become highly activated during aging, and it acts towards CNS and peripheral nervous system (PNS) insults combined. Caldeira et al. [57] have reported by *in vitro* experiment that the isolated microglial cell tends to show a reduced reaction in autophagic capability, chemotaxis, phagocytosis, and overall reactivity.

3. Microglia in Neurodegeneration

Microglial activation exacerbates the production of cytokines, chemokines, and other factors that trigger AD progression [58]. Not only do proliferative microglia correlate with disease severity in AD patients but also AD animal models [59]. Their gradual gathering and changing in

signaling prompt the cognitive decline; thereby, targeting microglia and their signaling pathways would be a potential therapeutic strategy.

By using a gene expression profile, a study identified a newer type of microglia that extended the existent microglia classification, and investigators in this inquiry yclept this molecular signature of disease-associated microglia (DAM, distinctive microglia subgroups) [24]. Interestingly, Krasemann et al. [24] showed that microglial neurodegenerative phenotype (MGnD) upregulated 28 inflammatory molecules and diminished the expression of 68 homeostatic microglial genes; in contrast, a large segment of these activities disappeared in the microglia-specific knockout of *APOE* in mice. These findings indicate that the *APOE* strongly persuades phenotypic switching in disease-related microglia and is upregulated through the vicinity of plaques. Moreover, MGnD microglia remarkably increased in miR-155 expression resulting in a notable upregulation of microRNA (miRNA) in microglia after extreme provocation with an insult, which leads to the release of proinflammatory molecules, including interleukin-6 (IL-6), interleukin-1β (IL-1β), nitric oxide synthase-2 (NOS-2), and tumor necrosis factor-alpha (TNF-α) [60]. In 5XFAD transgenic mice, elevation of *APOE*, *TREM-2*, and leukocystatin (*Cst7*) gene expression was associated with the transition from homeostatic microglia to DAM activation [61]. Previously, it has been demonstrated that DAM activation is tightly linked with the loss of microglial homeostatic genes such as purinergic receptor P2Y (P2RY12) and CX3CR1 [61].

In addition, Runt-related transcription factor 1 (RUNX-1), Sal-like 1 (SALL-1), T-cell-acute-lymphocytic leukemia protein-1 (TAL-1), and interferon regulatory factor 8 (IRF8) genes acquainted with microglia maturation and ramification are also influenced by AD pathology [60]. Usually, MGnD is a consequence of chronic manifestation of disease pathology and can easily differentiate between M1 and M2 microglia through the appearance of ApoE, TREM2, chitinase-3-like protein (Ym1), arginase 1 (Arg1) as well as the nonappearance of a homeostatic transcription factor, namely, early growth response protein 1 (Egr1), respectively [24]. In the AD brain, both the MGnD and DAM phenotypes are upregulated in the microglia and influenced mainly by *TREM2* expression [62, 63]. From these studies, the researchers propose that the *APOE-TREM2* signaling pathway is mainly accountable for the remodelling of the gene expression profile that prompts the MGnD phenotype in microglia [24, 61, 63]. While the relationship between the MGnD phenotype and aging is questionable, how microglia with advancing age are responsible for making this transition needs to be solved. Advance research is warranted for better understanding to elucidate the close relationship between the time-dependent *APOE-TREM2* signaling complex and the MGnD phenotype.

4. Activated Microglia and Alzheimer's Pathogenesis

Microglia have a dual role in AD pathogenesis; in AD, microglial detrimental effects are associated with proinflammatory mediators [64, 65]. Apparently, Aβ provoked the microglial activation, and deteriorated neuron-derived ingredients may exaggerate microglial neurotoxicity in AD [66]. Aβ subsists in several assembly forms, such as monomers, oligomers, and fibrils. However, from these three Aβ assemblies, only oligomeric Aβ (oAβ) and fibrillar Aβ (fAβ) have been implicated to microglial releases of proinflammatory mediators (Figure 1) such as cytokines (i.e., IL-1, IL-6, and TNF-α), chemokines (i.e., monocyte chemotactic-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1)), and reactive oxygen species (ROS) [67, 68].

The expression of nicotinamide-adenine-dinucleotide-phosphate-oxidase (NADPH oxidase) is stimulated to produce the ROS, which is correlated with the upregulation of AD [69]. fAβ is liable to microglial NADPH [70, 71], and activation of NADPH oxidase eventually leads to neurotoxicity. In fact, microglia produce extracellular ROS that has been directly harmful to neurons. Moreover, intracellular ROS act like a signaling molecule in microglia, which promotes the secretion of different proinflammatory cytokines and neurotoxic molecules [72].

Furthermore, glutaminase expression was disorganized by microglial activation; as a result, release of a large proportion of glutamate influenced excitoneurotoxicity through the N-methyl-D-aspartate (NMDA) receptor signaling pathway [73–75]. Previously, it has been demonstrated that persistent triggering of extrasynaptic NMDA receptors contributes to accelerated Aβ production [76]. Accumulating evidence supports that expression of Aβ itself disrupts the synaptic function, such as suppressing hippocampal long-term potentiating, the assistance of prolonged depression, and disturbance of synaptic plasticity [77, 78]. Hence, it is crucially important to examine the microglial neurotoxicity along with Aβ neurotoxicity. In addition, both the tau protein and Aβ pathology have been directly linked to the neuroinflammatory responses through the accumulation of reactive microglia and astrocytes, which are close to the amyloid deposits, an additional histological characteristic of AD [8, 79]. For example, in P301S tau, transgenic mice exhibit prominent microglial activation that ultimately disrupts hippocampal synaptic function [80]. Thus, microgliosis-induced hippocampal synaptic pathology may be the earliest expression of neurodegenerative tauopathies. Activated microglia can also reactivate astrocytes by releasing cytokines, including IL-1α, TNF-α, and C1q [81]. Reactivation of these astrocytes notably upregulates complement cascade genes, including C3, and fails to contribute to synaptogenesis and phagocytose synapses and myelin debris. In the prefrontal cortex of AD patients, nearly 60% of the astrocytes are C3-expressing astrocytes and may possibly cause neuronal injury [81]. During AD, reactive astrocytes interact with neuronal and nonneuronal (i.e., microglia and oligodendrocytes) cells by secreting feedforward signals and contributing to the vicious cycle that expedites neurodegeneration [82]. Although reactive astrocytes have both beneficial and harmful functions during AD, atrophic astrocytes (reduction of the surface area and volume of astroglial morphological profiles) might lose their homeostatic functions.

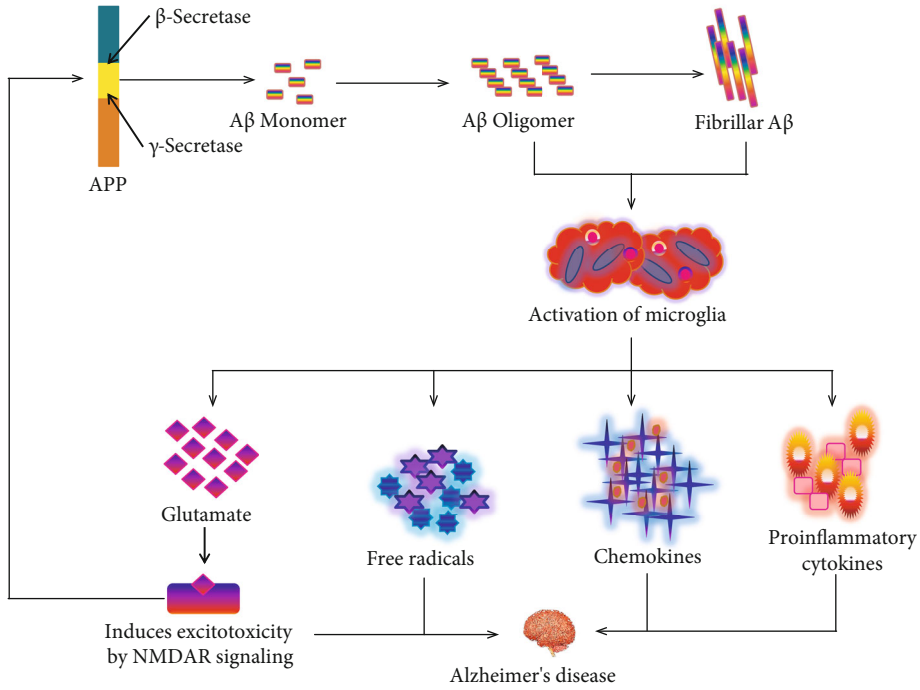

Figure 1: Role of Aβ in the activation of microglia to initiate Alzheimer's pathology. Aβ: amyloid beta; APP: amyloid precursor protein; IL-1: interleukin-1; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; MCP-1: monocyte chemotactic-1; MIP-1: macrophage inflammatory protein-1; HO: hydroxyl radical; H_2O_2: hydrogen peroxide; O_2: oxygen radical.

Microglia-neuron communication is bidirectional. Microglia-derived exosomes serve as a carrier for tau and Aβ in the brain. On the other hand, neuron-derived exosomes have similar effects on microglia. A study has shown that microglia act as scavengers by uptaking neuronal exosomes containing toxic proteins, including pTau and Aβ [83].

5. Microglia Receptors in the Amyloid Cascade of Alzheimer's Disease

5.1. Complement Receptors. Complement components (CRs) and their receptors are categorized as cell surface molecules on microglia that are located within or around Aβ cerebral plaques in AD [84]. Previously, it has been demonstrated that microglia not only express complement protein components such as complement component-1 (C1q) and complement component-3 (C3) but also precisely express complement receptors, including complement receptor type-1 (CR1), complement receptor type 3 (CR3), complement receptor type-4 (CR4), and complement component 5a receptor 1 (C5aR1), which support phagocytic uptake [85]. The imbalance of these complementary systems is correlated with the development of AD pathogenesis (Table 1). For instance, Aβ plaque formation was observed to be markedly increased with the suppression of these complement systems in the AD transgenic mouse model [86]. However, different proteins of the complement system and its analogous mRNAs are unregulated, resulting in Aβ-instigated inflammation, the emergence of senile plaque, and Aβ phagocytosis in AD patients [87]. C3 is denoted as a protein and an integral part of the complement system, which influences the phagocytosis of pathogens by interacting with the CR3 receptor. CR3 is also familiar as a macrophage-1 antigen and indisputably observed in microglia that have been upregulated by the AD brains [88]. In addition, both *in vivo* and *in vitro* studies have demonstrated that CR3 was responsible for the uptake and clearance of Aβ [89–91]. Likewise, this receptor is partially associated with Aβ-induced microglial activation and involved in Aβ-mediated microglia ROS generation [92], as stated in Figure 2. Furthermore, a study in AD mice showed that microglia were associated with synaptic pruning in a CR3-dependent pathway [93]. More clearly, oligomeric Aβ locally activated complement (i.e., C1q and C3) at vulnerable synapses, resulting in microglial engulfment of these synapses via C3/CR3 signaling. Nowadays, CR3 antagonists are widely accepted as potential therapeutics to treat AD owing to their potential to significantly decrease the Aβ-induced proinflammatory molecules and ROS in microglia [92].

C5a is a protein fragment that can generate highly proinflammatory molecules via activating the complement system [41]. It is also called a CD88 and is located on the surface of the microglial cell. CD88 is involved in microglial recruitment and activation; an elevated level of CD88 has been observed in microglia and appeared close to the amyloid plaques in the AD mouse brains [119]. In addition, coinvigoration of human monocytes with Aβ and C5a encourages the promotion of IL-1β as well as IL-6 secretion [120]; mitigating the destructive role of CD88 would be a potential strategy for AD pathogenesis. Therefore, Fonseca et al. [97] conducted a study to assess the efficacy of this receptor

Table 1: Outline of microglia receptors and their function in Alzheimer's disease.

Microglia receptors	Functions in Alzheimer's disease	References
Complement receptors (CRs)	(i) Phagocytic uptake (ii) Microglia activation (iii) Proinflammatory molecule generation (iv) Aβ clearance	[89–92, 94–97]
Toll-like receptors (TLRs)	(i) Proinflammatory mediator generation (ii) Aβ clearance (iii) Microglia activation (iv) Synaptic plasticity (v) tau phosphorylation	[15, 98–103]
Scavenger receptor type-A (SR-A)	(i) Aβ internalization and clearance (ii) Inflammatory response (iii) Maintain microglia immune response	[104–107]
Cluster of differentiation 36 (CD36)	(i) Microglia recruitment (ii) Inflammatory response (iii) Activation of Aβ phagocytosis (iv) Modulates microglial Aβ42 phagocytosis	[108–111]
Receptor for advanced glycation end products (RAGE)	(i) Microglia activation (ii) Stimulate IL-1β (iii) TNF-α production (iv) Intensify oxidative stress	[112–116]
Triggering receptor expressed in the myeloid cell 2 (TREM2)	(i) Aβ clearance (ii) Regulates microglial mammalian target of rapamycin (mTOR) activation and metabolism (iii) Balanced microglial autophagy	[117, 118]

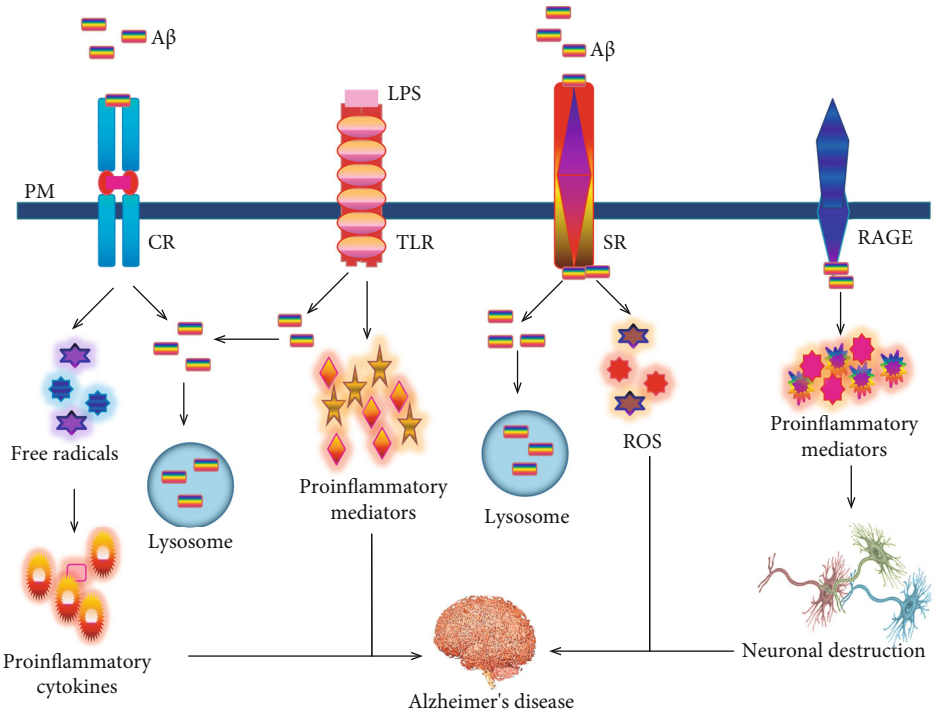

Figure 2: The linkage of microglia receptors in the pathogenesis of Alzheimer's disease. CR3 is responsible for the Aβ-induced microglial activation and involved in Aβ-mediated microglia free radical generation as well as uptake and clearance of Aβ. TLR2 is implicated in the generation of the inflammatory response. On the other hand, TLR4 (i.e., stimulated with LPS) is associated with the clearance of Aβ. Microglia cells showed an increase in Aβ uptake. The binding of Aβ to SRs internalizes Aβ and could activate inflammation responses and generate reactive species. Microglia RAGE-Aβ interaction triggers the genesis of proinflammatory molecules that causes neuronal destruction. PM: plasma membrane; Aβ: amyloid beta; CR: complement receptor; LPS: lipopolysaccharide; TLR: Toll-like receptor; SR: scavenger receptor; RAGE: receptor for advanced glycation end products; ROS: reactive oxygen species.

antagonist, which markedly attenuated Aβ plaques, reduced glial triggering, and ameliorated context-dependent memory in double transgenic AD mouse models (Table 2).

Although accumulating evidence indicates the complement system manifested detrimental effects, a few data claimed that it has beneficial effects too in AD. For instance, C3-deficient APP mice showed an elevated level of Aβ in the brain area linked with notable neuronal damage [89]. More interestingly, higher expression of C3 mRNA levels is linked with a depletion in Aβ deposition in hAPP/TGF-β1 transgenic mice [121]. Overall, activation of these receptors might encourage the Aβ clearance, therefore eventually decreasing the Aβ accumulation in the AD. Still, many issues remain unsolved, so future studies are warranted to expurgate the molecular mechanism of the complement system in the brain and evaluate its suitability to the design and development of novel AD treatments.

5.2. Toll-Like Receptors. In 1997, Toll-like receptors (TLRs) were first identified as membrane proteins found in different types of cells, such as microglia and astrocytes [124, 125]. Although in mammals, there are 12 TLRs that have been described, only TLR2 and TLR4 can recognize Aβ [126]. However, its activation stimulates several signaling pathways; as well as, the secretion of several cytokines, nitric oxide (NO), and ROS [98]. Surprisingly, animal and human brain microglia expressed among the TLRs 1-9 and maxima of these receptors were responsible for microglial activation and neurotoxicity [125, 127]. For example, aged APP23 transgenic mice showed an upregulation of TLR-2, TLR-4, TLR-5, TLR-7, and TLR-9 mRNA levels in plaque-related brain tissue [128]. Studies have demonstrated that TLRs stimulate the intracellular cascade that leads to either release of proinflammatory mediators or the uptake and clearance of Aβ [99, 102]. Likewise, TLR2 involvement in the activation of microglial proinflammatory signaling to Aβ has been shown in Figure 2. Both AD patients and AD murine models found an increase in mRNA levels for TLR2 in the brains [129, 130]. Additionally, it has been reported that deficiency of TLR2 promotes a reduction in both spatial and nonspatial memory [123]. Interestingly, knockdown of TLR2 mice has disclosed a depletion of Aβ-induced manifestation of proinflammatory molecules (i.e., TNF-α, iNOS, IL-1β, and IL-6) and integrin markers (i.e., CD11a, CD11b, and CD68) in microglia [99]. Likewise, Liu et al. [101] have demonstrated that TLR2 deficiency suppressed Aβ-induced inflammatory signaling and improved Aβ internalization by phagocytosis in cultured microglia and macrophages. So suppression of TLR2 would be a powerful scheme that could markedly dwindle the inflammatory response and notably enhance the Aβ clearance, consequently slowing the AD pathogenesis.

TLR4 can recognize LPS by microglia; previous studies have identified its influences on stimulating the microglia-Aβ activation [131]. For instance, an activated murine microglia cell demonstrates that TLR4 contributes to Aβ-induced microglial neurotoxicity combined with a CD14 and myeloid differentiation protein-2 (MD2) [131]. In an *in vitro* experiment, microglia cells invigorated with LPS (i.e., a TLR4 ligand) showed an upregulation of Aβ uptake [102], as shown in Figure 2. In addition, both *in vivo* and *in vitro* studies on an LPS-deficient response have revealed that microglia increased the Aβ load and decreased Aβ uptake [102]. Moreover, in early stages, the TLR4-mutated AD animal model expressed a deficiency of spatial learning and increased levels of Aβ42 in the brain [122]. Altogether, roundup evidence on TLR2 and TLR4 indicates that depending upon diverse microglial phenotypes, these receptors have a complex role in AD. However, consolidated evidence strongly suggests that activation of TLR2 and TLR4 contribute to AD progression, and their inhibition may suppress AD pathogenesis [132]. Maybe these receptors show their beneficial effects in the early stages of AD, and their opposite role is exhibited in the late stages of AD due to diverse microglial phenotypes. Therefore, microglial TLR2 and TLR4 represent an acceptable target for therapeutic intervention within the disease progression, and targeting them could increase Aβ phagocytosis or reduce inflammatory responses [133–135].

5.3. Scavenger Receptors. Two kinds of scavenger receptors (SRs) have been identified in the CNS. Scavenger receptor type-A (SR-A) is manifested on microglia and astrocytes, whereas scavenger receptor type-B (SR-B) receptors are manifested on microglial and endothelial cells [108]. Microglial adherence via SR-A binding to fibrillar Aβ causes microglial immobilization, the genesis of ROS, and secretion of cytokines [70]. Both of these SRs could bind and internalize Aβ (Figure 2), inducing an inflammatory response that leads to AD pathogenesis [104]. Furthermore, both the SR-AI expression levels and Aβ clearance have been attenuated by prolonged preservation of microglia activation [106]. In addition, SR-AI deficiency with a presenilin1 (PS1)/APP transgenic mouse brain showed that increased levels of Aβ deposition correlated with an increase in mortality [11].

CD36 is a pattern recognition receptor (PRR) found on many different kinds of cells. This receptor comprehends not only exogenous molecules, for example, microbial elements [136] but also endogenous molecules, such as low-density lipoproteins (LDL), oxidized phospholipids (oxPCCD36) [137], programmed cell death-related cells, and Aβ [138]. CD36 is responsible for the development of several diseases, including AD [139]. Furthermore, CD36 interacts with Aβ by microglia to generate ROS [110] and activation in response to fAβ [108, 110, 140]. For instance, reducing the expression of cytokine and chemokine such as monocyte chemoattractant protein-1 (MCP-1), IL-1β, macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP1β), macrophage inflammatory protein-2 (MIP-2), and TNF-α has been seen in macrophages and microglia from CD36-deficient mice vivified with fAβ [110]. However, in human brains, CD36 was observed at an overexpressed level with Aβ deposits, but without Aβ deposition, CD36 has not been detected in healthy brains [141]. Moreover, CD36 configures complexes with other PRRs to bind to fibrillar proteins.

CD163 is an unclassified SR that is expressed on mature tissue macrophages and is involved in hemoglobin-haptoglobin clearance from the blood [142]. CD163

Table 2: Microglia in various Alzheimer's disease preclinical models.

Species/studied material	Experimental model	Effects	References
Complement C3-deficient APP transgenic mouse (APP; $C3^{-/-}$)	Mouse model of AD	(i) Increased Aβ levels (ii) Elevation of fibrillar amyloid plaque burden	[89]
Homozygous C3-deficient and Mac-1-deficient mice ($C3^{-/-}$; Mac-$1^{-/-}$)	Mouse model of AD	(i) Implicated in the phagocytosis and removal of fAβ by microglia	[91]
hAPP transgenic mouse	Aβ-induced neurotoxicity	(i) Reduced Aβ accumulation	[121]
TLR2 knockdown mice	Aβ42-induced neuroinflammation	(i) Suppressed proinflammatory molecules and integrin markers in microglia	[100]
Mutation of TLR4 in C3H/HeJ mice	Aβ-mediated cognitive dysfunction and neurotoxicity	(i) Reduced microglial activation (ii) Increase Aβ deposits	[122]
CD36-deficient C57BL/6J mice	Nitro blue tetrazolium induced ROS generation	(i) Decreased microglial recruitment to sites of fAβ (ii) Reduced the production of ROS, TNF-α, IL-1β, and several chemokines	[110]
RAGE overexpressed mAPP transgenic mice	Mouse model of AD	(i) Increased the production of IL-1β and TNF-α (ii) Enhanced the infiltration of microglia as well as astrocytes (iii) Decreased acetylcholine esterase activity and causes Aβ accretion	[115]
Mutation of TLR4 in Mo/Hu APPswe PS1dE9 mice	Amyloidogenesis	(i) Increases in diffuse and fAβ deposits (ii) Decreased Aβ uptake by microglia	[102]
PS1-APP transgenic mice	Aging induced Aβ deposition	(i) Early microglial enrollment fosters Aβ clearance (ii) Aβ-mediated inflammation reduces microglial Aβ clearance	[106]
Aged APP23 transgenic mice	Aβ induced neuroinflammation	(i) TREM2 is increased in microglia associated with amyloid plaques (ii) Lack of extracellular amyloid clearance due to TREM2 signaling	[117]
AD-mutant hAPP transgenic mice	Complement receptor and microglia mediated synaptic loss early in AD	(i) Inhibition of CR3 decreases phagocytic microglia (ii) Adult brain microglia engulf synaptic material when exposed to soluble Aβ oligomers	[93]
Triple transgenic mice that are deficient in TLR2 ($TLR2^{-/-}$)	Aβ42-induced memory impairments	(i) Accelerated spatial and contextual memory impairments (ii) Increased levels of Aβ	[123]
APP/PS1/SR-$A^{-/-}$ mice	Aβ induced cognitive decline and neuroinflammation	(i) Increased neuroinflammation (ii) Elevated Aβ accumulation (iii) Increased cognitive impairment	[107]

engagement caused macrophages to produce proinflammatory mediators, indicating that CD163 is involved in macrophage activation [143]. Fabriek et al. [144] also reported CD163 functions as an innate immunological sensor and modulator of local inflammation in the host's defense against both gram-positive and gram-negative bacteria. Interestingly, CD163 was found to be expressed on microglia in the brains of patients with HIV-associated dementia [145]. However, whether CD163 is involved in AD pathogenesis is still elusive.

5.4. Receptor for Advanced Glycation End Products. The receptor for advanced glycation end products (RAGE) is a multiligand receptor and a compelling factor in aging that identifies the Aβ peptides [146]. Previously, it has been observed that Aβ provokes nuclear factor kappa light chain enhancer of activated B cell (NF-κB) activation in several cells and stimulates the release of proinflammatory mediators by the dealings with RAGE [147, 148].

Different experimental data disclosed that microglial RAGE-dependent molecular signaling drives Aβ-induced inflammatory response and neuronal damage in the AD [112–114, 149]. In particular, the experimental result proposes that the p38 mitogen-activated protein kinase (MAPK) signaling pathways engage in the activation of microglia through the interaction between Aβ and RAGE receptor [113, 115]. Fang et al. [115] have documented microglial RAGE in the pathogenesis of AD and proposed that refraining of the RAGE signaling pathway may be a quintessential target for reducing the secretion of proinflammatory molecules like TNF-α and IL-1β after Aβ stimulation in the AD. Microglia RAGE-Aβ interaction stimulates to upregulate the proinflammatory response as a consequence; the neuronal destruction that directly influences a shortage in learning and memory is mentioned in Figure 2. Further exploration has been suggested to ascertain small molecules for the blocking of Aβ-RAGE interaction, which would be a possible therapeutic stratagem to deal with most devastating AD pathogenesis.

6. Microglia in the Spread of Tau Pathology in Alzheimer's Disease

The hyperphosphorylation and accumulation of microtubule-associated protein tau (MAPT) form the initial event before neurodegeneration [150]. In humans, neuroinflammation is positively linked with tau pathology and is involved in the production of tau hyperphosphorylation, accumulation, and neurodegeneration [151, 152]. In the P301S animal model of tauopathy, it has been shown that microglial activation is the earliest manifestation of tau pathology [80]. Notably, in this study, they administered FK506 (i.e., an immunosuppressant drug), which reduced the microglial activation and augmented the lifespan of tau (P301S) transgenic mice [80]. Later, Maphis et al. [19] demonstrated that activated microglia played a pivotal role in the proliferation of tau. Afterwards, Bolós et al. [153] reported that microglia phagocytose the tau. However, how microglia induced tau pathology is yet to be confirmed.

Interestingly, an in vivo humanized mouse model of tauopathy (hTau) showed that either chemical compound or genetically induced microglial triggering markedly manifested tau pathology and behavioral malformation [154]. Furthermore, in hTau mice, deficiency of microglia-specific CX3CR1 evolved in triggered microglial activation as a result of increased tau pathology and impaired working memory [154]. This effect is arbitrated through the IL-1/p38 MAPK signaling pathway. Another study showed that deleting CX3CR1 in hAPP mice promoted the expression of inflammatory mediators and enhanced plaque-independent neuronal abnormality as well as cognitive deficits [155]. The CX3CL1/CX3CR1 signaling pathway is an important neuron and microglial communication [156]. A study demonstrated that nonappearance of CX3CR1 weakens the microglial internalization of tau, which leads to AD progress [157]. Accumulating studies indicate that microglia-allocated neuroinflammation increases the tau pathology as a consequence of neurodegenerative disease. In hTau mice, Maphis et al. [19] evaluated that depending on the different disease stages, CX3CR1 deficiency is responsible for the onset and development of tau pathology. They suggest that these reactive microglia can influence the development of the tau pathology and be consistent with the propagation of pathological tau in the brain. In addition, a study reveals that lacking microglial TREM2 results in exacerbated tau pathology and a profound dysregulation of stress-related kinase pathways in a humanized mouse model of tauopathy [158]. On the other hand, TREM2 reduces neuronal tau hyperphosphorylation by reducing the microglial inflammatory response [159]. During the pathological investigation of human brains, it has been found that microglia morphologically degenerated and were associated with tau pathology [160]. These morphological changes are suggested to result from microglial senescence and chronologically precede the spread of tau pathology [161, 162].

7. Microglial Activation in Alzheimer's Stage

7.1. Activated Microglia in Early-Onset Alzheimer's. The amyloid cascade-neuroinflammation hypothesis characterized as an abnormal production of Aβ owing to the redundancy of Aβ synthesis or a dysfunctioning of Aβ clearance is the paramount causality of the AD, which consequently stimulates neuroinflammation-induced neuronal loss [163, 164]. Therefore, neuroinflammation is noticed as a critical factor in the development of AD pathogenesis [165]. In addition, activated microglia can be either proinflammatory or anti-inflammatory.

In AD at its early stages, it has been proposed that the initial microglial activation may have a beneficial function through the clearance of the amyloid and releasing potential nerve growth factors [166]. On the other hand, due to the failure of this process hence to promote the Aβ aggregation or other lethal products, therefore, activation of proinflammatory phenotypes leads to a rapid destruction of the neurons. However, the genetic data from GWAS propose microglial activation to be able to execute several critical functions in the early stages of AD and autonomous amyloid

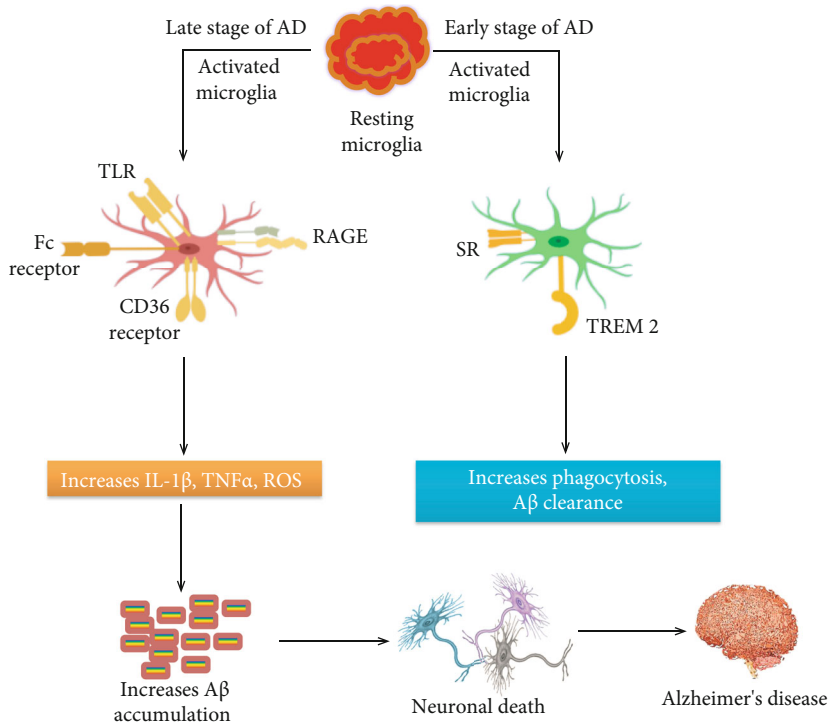

Figure 3: Possible mechanisms of action of activated microglia in different early and later stages of Alzheimer's disease. In the early stages of AD, activated microglia may increase Aβ clearance through TREM2 and scavenger receptors. On the other hand, in the late stage of the disease, continuous microglial activation induced by Aβ through various receptors triggers a vicious cycle of microglial activation, neuroinflammation, and Aβ buildup that leads to AD. AD: Alzheimer's disease; TLR: Toll-like receptor; RAGE: receptor for advanced glycation end products; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; ROS: reactive oxygen species; SR: scavenger receptor; TREM2: triggering receptor expressed on myeloid cell 2

pathology [167, 168]. Although epidemiological analysis has demonstrated that people who take nonsteroidal anti-inflammatory drugs (NSAIDs) have an inferior frequency of AD, randomized control trials have not shown the effectiveness of these NSAIDs in subjects with later onset of AD [169]. Recently, one study hypothesized two particular stages of microglial activation in the AD trajectory, an early anti-inflammatory phase and an advanced proinflammatory phase [170]. In this case, targeting antimicroglial medications would be most favorable to protect against the battle of the proinflammatory phenotype in the advanced phase of this disease. In the early phase of AD, microglial activation is able to alleviate Aβ aggregation by augmenting its phagocytosis, clearance, and degradation properties [171, 172]. For instance, an investigation of amyloid plaques by electron microscopy demonstrated that microglia are efficiently engulfing Aβ, and Aβ appeared in the endosome-like cellular domain [173].

7.2. Activated Microglia in Late-Onset Alzheimer's. In late-onset AD (LOAD), microglia have been deprived of their beneficiary function due to a tenacious production of proinflammatory mediators [174]. A study has shown that the amyloid plaque burden upsurges with aging in human patients, indicating the relatively ineffective phagocytic potential of microglia [175]. In human AD, Aβ42 immunization improves the function of microglia by intensifying their phagocytic activity [176]. Based on the microglial dysfunctioning notion, there is a loss in microglial neuroprotective activity in AD, rather than an increase in an inflammatory role [177]. Previously, it has been reported that the microglial phagocytic capabilities are shifted with aging and similarly decrease this feature in neurodegenerative diseases. Likewise, these senescent (i.e., biological aging) microglia are linked with the onset of sporadic AD [178]. Furthermore, recent studies on TREM2 also ascertain both early and late stages of microglial activation during the AD trajectory [179, 180]. TREM2 expressed in microglia is supposed to link with microglial activation. Even though a few studies indicate opposed effects for TREM2 levels in AD, lately, a study reported the level of soluble TREM2 (sTREM) directly linked with the early and delayed stage of AD [181, 182], where the peak beneficial role of TREM2 has observed in the early stage and later stage; its salutary effect gradually decreased (Figure 3).

8. Microglial Deterioration in Alzheimer's Patients

The activated microglial response has been extensively explored in AD brain regions by comparatively exalted Aβ subjects or in Aβ-rich transgenic models [183–185]. Fascinatingly, Aβ accumulation and neurofibrillary tangles (NFTs) do not appear in similar anatomical locus; in this sense, a direct pathogenic connection between amyloid

plaques and neurodegenerative diseases is still elusive [186, 187]. In fact, cognitive disability is not compatible with an overabundance of amyloid plaque, even so with the presence of neurofibrillary pathology explicit as tau-positive morphology, including unmyelinated axons in the nervous system so-called neuropil threads, NFTs, and neuritic plaques [188, 189]. Additionally, an unambiguous determination of microglial activation in the human brain is extremely complicated since there is no effective biomarker for differentiating between activated and nonactivated cells. It is also surprising that microglial cells become progressively dysfunctional with aging in the human brain that displays morphologically senescence rather than activation, like fragmented cytoplasmic processes [190]. The identification of senescence microglia has imparted new aspects on the possible implication of microglia in aging-associated neurodegeneration; for example, aging causes loss of notable microglial cell function involved in the reduction of microglial neuroprotection [190, 191]. A study evaluating the microglial reaction in postmortem hippocampal human tissue demonstrated that microglia underwent a noticeable degenerative process in the dentate gyrus (DG) as well as CA3 of Braak V–VI samples, likely to be the case linked with the accumulation of soluble pTau [160].

Not only are microglial cells able to protect the synaptic integrity [192] but also they contribute to the learning ancillary synaptic formation [193]. Moreover, microglia induce Aβ-phagocytosis [194, 195] and senile plaque compaction and limit the Aβ toxicity [196, 197]. Furthermore, microglia contribute to removing depreciated neurons as well as neuronal stuff, for example, paranormal synaptic terminals or axonal demyelination. In this context, deficits in colony-stimulating factor 1 receptor (CSF1R) or TREM2 are correlated with a rare group of neurodegenerative disorder, for example, adult-onset leukoencephalopathy with axonal spheroids (i.e., characterized by excessive demyelinating lesions in the cerebral white matter) or Nasu-Hakola disease (i.e., characterized by multiple bone cysts linked to neurodegeneration), respectively [198–200]. These studies, together with Sanchez-Mejias [160] data, strongly indicated microglial pathology resulting in a deficient immunoprotection in DG and CA3 that leads to progressive AD pathology and cognitive damage. Furthermore, TREM2-knockout models show dystrophic microglial cells [201], shortages in microglial survival, and worsening in AD pathology [22].

Accumulating evidence demonstrates that AD is associated not only with microglial activation but also with microglial senescence, which might be considered the degeneration of these cells continuously [190]. These findings suggest that NSAIDs have become incapable of preventing or decreasing neurodegenerative disease like AD. Surprisingly, they reconstructed the conception regarding AD pathogenesis far away from inflammation-related impairment and proximate to an uninvestigated area of neuroscience, for instance, activities or events that can destroy microglial cells. Incredibly, it has become crystal clear that senescence microglia are responsible for age-related telomere length (TL) shortening [202, 203]. In addition, shortened TL in peripheral blood leukocytes is further recognized as early jeopardy of dementia [204]. Since microglia are indispensable for providing neuroprotection [191], aging-associated loss of a microglial protective role in neurodegenerative disease is likely to have detrimental repercussions for neurons.

9. Conclusion

Nowadays, studies started focusing on microglia to better understand the functional role of microglia in changing the progression of AD. Microglial cells are dynamic and reactive and change their surrounding environment rapidly, resulting in either proinflammatory or anti-inflammatory states. It has become apparent that microglia not only produce neurotoxic products but also need for phagocytic clearance of neurotoxic proteins associated with AD. The randomized control trials that employed nonspecific anti-inflammatory agents have not appeared to be significant in mitigating disease, possibly because of the inhibition of indispensable phagocytic functions that accumulate toxic proteins. Furthermore, depending on the clinical environment, microglia phenotypes may have a negative or positive effect. In fact, the ultimate beneficial role of TREM2 has been observed in the early stage and later stage, and its beneficial effect gradually decreased. AD pathogenesis is dependent on microglial cells and their receptors. Therefore, targeting microglial receptors to maintain microglial homeostasis would be a potential therapeutic strategy in AD.

Conflicts of Interest

The authors proclaim no conflict of interest.

Authors' Contributions

MSU conceived the original idea and designed the outlines of the study. DMS, MSU, MTK, and SH wrote the draft of the manuscript. MSU and DMS prepared the figures for the manuscript. AP, ISA, GMA, MMA-D, and GhMA performed the literature review and aided in revising the manuscript. All authors have read and agreed to the published version of the manuscript.

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Review Article

Galectin-3: A Novel Marker for the Prediction of Stroke Incidence and Clinical Prognosis

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Stroke, whether ischemic or haemorrhagic, is one of the main causes of mortality and disability all over the world, which entails huge burdens in both healthcare environments as well as social and economic aspects of life. Therefore, there is a continuous search for novel reliable biomarkers that can enhance the recognition of stroke events in a timely manner and predict the clinical outcomes following a stroke event. Galectins are a group of proteins expressed by many types of cells and tissues including vasculature, certain immune cells, fibroblasts, and gastrointestinal epithelial cells. These proteins vary in their structure and configuration according to their type and have a diversity of functions according to the type of tissue they are expressed in. Among these proteins, a few studies investigated mainly the roles played by galectin-1 (Gal-1) and galectin-3 (Gal-3) in the molecular mechanisms of atherosclerosis and in brain tissue remodeling after a stroke event. In this review, we present an updated overview of the current understanding of Gal-3's functions and implications in stroke occurrence and the response of the brain tissue to stroke events, which may be a key to its utility as a predictor of stroke incidence and clinical prognosis in the future.

1. Introduction

Stroke is one of the leading causes of disability and mortality worldwide. As defined by the American Heart Association, the term "stroke" encompasses any acute neurological deficit originating from a vascular origin [1]. This definition includes cerebral ischemia, intracerebral haemorrhage, and subarachnoid haemorrhage. Thus, stroke can be classified into two major categories, ischemic stroke —accounting for almost 85% of stroke incidences— and haemorrhagic stroke [1, 2]. Due to the rising incidence rate as well as the significant healthcare, social, and economic burdens of stroke, stroke events have been intensively studied, and several modifiable risk factors of stroke have been identified including hypertension, atherosclerosis, cardiovascular disease, diabetes, and smoking [3].

Despite the fact that patients with such risk factors are considered to be at a higher risk of stroke than the general

population, there is still a need for reliable biological markers which can accurately predict the diagnosis of stroke in suspected patients, especially in populations at risk. Additionally, these markers would be of much benefit if they can further predict the clinical prognosis following a stroke event, which typically factors in the management plans of stroke patients. To meet this need and improve the standards of care, several biochemical markers are currently under investigation to assess their potential ability to predict stroke incidence. Of these markers are galectins, a group of glycan-binding proteins known for their role in intracellular interactions and immune regulation. In this literature review, we are discussing the molecular basis of galectins' functions under normal physiological conditions, their utility as predictive and/or prognostic makers following stroke, and how they may alter stroke management.

2. Biochemistry of Galectins and Their Physiological Roles

Amongst the many complex glycan-protein interactions which govern numerous intracellular processes, galectins serve a variety of important functions [4]. Galectins are abundantly expressed in many tissues, including gastrointestinal epithelial cells [5, 6], the adventitia of blood vessels [7], fibroblasts [8], thymocytes [9], leukocytes [10], and neuroglial cells [11]. Numerous biological functions are attributed to the expression of galectins including the recognition of cell damage, adherens junction formation, and cell growth regulation. Additionally, they are known to play vital roles in immunological processes including inflammation, apoptosis, phagocytosis, cellular activation, and migration [12].

All galectins share a homologous carbohydrate recognition domain (CRD) which has an affinity to β-galactosides, and they can be classified into three subfamilies based on the organization of their CRD: (1) prototypical galectins (Gal-1, 2, 7, 10, 13, and 14) with only one CRD, (2) chimeric galectins (Gal-3) with one CRD and an N-terminal extension, and (3) tandem-repeat galectins (Gal-4, 8, 9, and 12) with two nonidentical CRDs connected by a short peptide chain [4]. As a result of such differences in their structure, galectins can form quaternary structures, with prototypical galectins forming homodimers, tandem-repeat galectins forming dimers, and chimeric galectins forming higher-order oligomers [4]. These different structures determine the physiological functions of the different types of galectins. Currently, 16 galectins have been identified in mammals, 12 of which have been isolated in humans [13, 14] (Table 1). Only six have been identified in the human brain (Gal-1, 2, 3, 8, 9, and 14) [15], with Gal-3 being the most frequently investigated in clinical settings for stroke prediction and prognosis. As a result, this review will focus primarily on the implications of Gal-3 in the pathogenesis and outcome of stroke.

3. The Pathophysiological Role of Galectins

3.1. The Role of Galectins in the Pathogenesis of Stroke. Atherosclerosis is a complex multifactorial pathological process that might lead to an array of cerebrovascular diseases. Over the years, galectins have been shown to be intimately involved in this process. For instance, Gal-1 expression has been positively correlated with the proliferation of smooth muscle cells in specimens obtained from atherosclerotic blood vessels [16]. A potential mechanism of this process is the ability of Gal-1 to bind to lipoprotein (a) (Lp(a)) [17], leading to its accumulation in arterial walls. Lp(a), in turn, triggers smooth muscle proliferation [18, 19] which is a main component of the atherosclerotic process [20] (Figure 1).

There is also evidence of Gal-3's involvement in the atherosclerotic process [21], as shown by its upregulation in human atherosclerotic plaques [22] and the beneficial effects reported in animal studies following inactivation of Gal-3 gene [23]. In apolipoprotein E- (ApoE-) deficient mouse models, when compared with mice with active Gal-3 expression, mice in which Gal-3 gene was knocked out showed a decreased number of the atherosclerotic lesions and reduced amounts of perivascular inflammatory infiltrates [23]. Gal-3 may exert its atherogenic effects via several mechanisms which include mediating the differentiation of monocytes to macrophages and subsequent transformation to foamy macrophages [24, 25]. These foamy macrophages are considered to be the pathological hallmark of atherosclerotic lesions [26]. Moreover, by acting as a chemoattractant when released by foamy macrophages, Gal-3 may enhance the recruitment of more inflammatory cells to the atherosclerotic plaque [27]. Besides, it might be implicated in the transformation of vascular smooth muscle cells into macrophages and the cellular uptake of oxidized low-density lipoproteins (ox-LDL) and advanced glycation end products leading to the production of more foam cells [25]. It can also exacerbate the inflammatory process of atherosclerosis through the activation of the β_1-RhoA-JNK signaling pathway [28], which ultimately aggravates endothelial injury [28].

The intimate association between galectins and the various aspects of atherogenesis, an essential risk factor for cerebrovascular events, is precisely what drove a number of researchers [29–31] to investigate the potential value of measuring galectin levels as a potential method of predicting stroke occurrences and other adverse vascular events.

Furthermore, galectins are known to have immunomodulatory effects, with Gal-3 being the most studied galectin in this regard. Gal-3 is the only chimeric galectin expressed by human cells, suggesting that its quaternary structure might play a role in determining its diverse range of functions. It is ubiquitously produced by various tissues including epithelial cells, cardiac myocytes, vascular endothelial cells [32], leukocytes [33], and glial cells [34]. De Giusti et al. [35] reported upregulation of Gal-3 expression by activated microglia and astrocytes in mice with induced encephalitis, bringing to light the possible involvement of Gal-3 in the pathophysiology of central nervous system (CNS) diseases.

Microglial cells are responsible for the immune reactions in the CNS and respond to brain injury in a graded manner known as microglial activation: a process that involves cell migration and proliferation leading eventually to an inflammatory response with the release of cytokines and other

Table 1: Structure and function of galectins found in humans [14, 69].

Galectin	Subfamily	Function	Main tissue distribution
Gal-1	Prototypical	Regulation of apoptosis Cellular proliferation and differentiation	Nonspecific
Gal-2	Prototypical	Unknown	Gastrointestinal Urogenital
Gal-3	Chimeric	Immunomodulation and recognition of cell membrane damage	Macrophages Fetal membranes Gastrointestinal
Gal-4	Tandem-repeat	Adherens junction formation	Gastrointestinal
Gal-7	Prototypical	Regulation of apoptosis Control of cell growth	Skin Oesophagus
Gal-8	Tandem-repeat	Recognition of cell membrane damage Restricts infection by viral and bacterial pathogens	Nonspecific
Gal-9	Tandem-repeat	Immunomodulation Bactericidal	Gastrointestinal
Gal-10	Prototypical	Immunoregulation Forms Charcot-Leyden crystals in eosinophils	Bone marrow
Gal-12	Tandem-repeat	Regulation of apoptosis	Adipose Breast
Gal-13	Prototypical	Regulation of apoptosis*	Placenta
Gal-14	Prototypical	Regulation of apoptosis*	Placenta
Gal-16	Monomer	Regulation of apoptosis*	Placenta

*Strong regulator of T-cell apoptosis.

inflammatory markers [36]. In this regard, microglial cells play a dual role in the brain's response to injury; firstly, they are responsible for the release of cytokines that promote cell death. Secondly, they produce trophic factors stimulating cellular proliferation and mediating phagocytosis, both of which are processes that allow for healing without further tissue damage [37, 38]. In addition, Barguillos et al. [39] have shown that the release of Gal-3 by microglia exerts a proinflammatory effect by acting as a ligand for Toll-like receptor 4 (TLR 4), with depletion of Gal-3 attenuating the inflammatory response. The protective effect attained by Gal-3 knockout was also demonstrated by Doverhag et al. [40] in mouse models of hypoxic-ischemic brain injury, whereby Gal-3 deletion reduced oxidative stress, matrix metalloproteinase, and overall brain injury.

On the one hand, Gal-3 is coexpressed alongside insulin growth factor-1 in microglial tissue in response to ischemic injury, where Gal-3 acts not only as an activator of microglia but also as a modulator of injury-induced microglial proliferative response [41]. Gal-3 is secreted extracellularly under the effect of interferon gamma (a proinflammatory cytokine) where it acts as a regulator of microglial and astrocytic response by modulation of JAK-STAT signaling [42] (Figure 2). Such an increase in the expression of Gal-3 was proven to take place during the first few days following a stroke event [43]. Gal-3 deficiency was found to worsen ischemic injury, suggesting that Gal-3 interactions might contribute to the resolution process [41] which might be explained by the loss of Gal-3's regulatory role of microglial activation and proliferation in response to ischemic brain injury. The anti-inflammatory effects of Gal-3 on ischemic lesions were further corroborated when delayed administration of recombinant Gal-3 was found to exert neuroprotective effects as it mediates a shift in the microglial activity away from the proinflammatory subtypes [44].

On the other hand, it was reported that Gal-3 released after acute inflammation, induced by intranigral lipopolysaccharide injection, acts as a Toll-like receptor 4 (TLR4) ligand. By binding to TLR4 on microglial cells, Gal-3 activates microglia into the proinflammatory variant and thus propagates inflammation and exacerbates cell damage [39]. The proinflammatory action of Gal-3, in a different type of injury (brain trauma), was also mediated by Gal-3's ability to bind to TLR4, and administration of antibodies against Gal-3 was found to be neuroprotective in this setting [45]. Dong et al. [46] reported similar findings, where Gal-3 gene knockout reduced the levels of proinflammatory cytokines and had an overall neuroprotective effect in the setting of ischemic brain injury.

In addition to its influence on glial cells, Gal-3 might also be involved in tissue remodeling and angiogenesis following ischemic strokes. Known mediators of angiogenesis and remodeling include matrix metalloproteinases (MMP), angiopoietin, and several growth factors, the most notable of which are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). In vitro studies suggested that Gal-3 acts in a VEGF-dependent manner to promote angiogenesis. It can do this via its chimeric structure. The interaction between the N-terminal CRD on Gal-3 and $\alpha v\beta 3$ integrin, which leads to integrin clustering, suggests that Gal-3 can trigger VEGF and bFGF to stimulate angiogenesis via an integrin-dependent signaling pathway

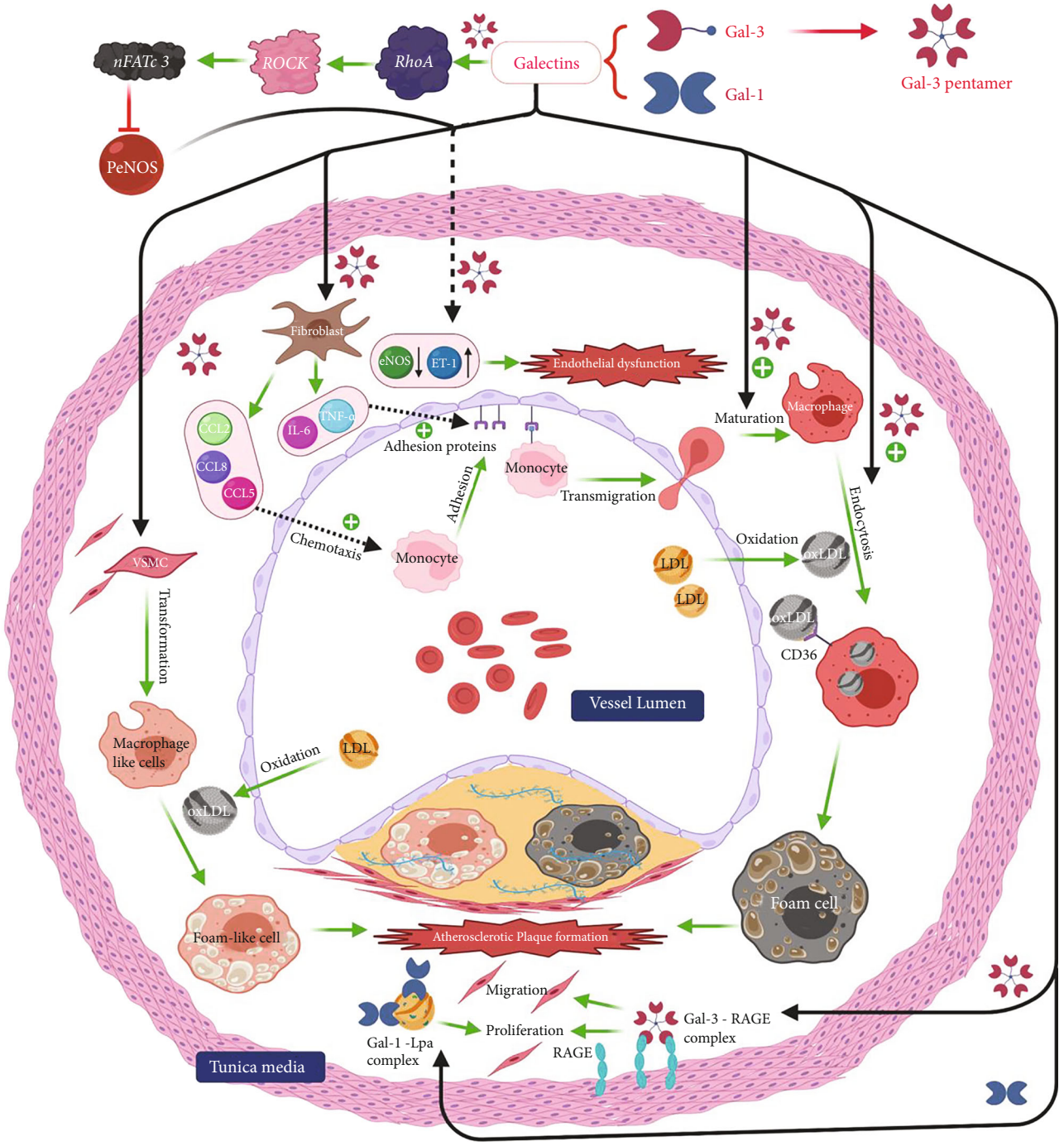

Figure 1: Demonstrates mechanisms and different pathways by which galectins can participate in the pathological process of atheromatous plaque formation as well as the induction of endothelial injury within blood vessels. RhoA: Ras homolog family member A; ROCK: Rho-associated protein kinase; nFATc3: nuclear factor of activated T-cells cytoplasmic 3; LDL: low-density lipoprotein; PeNOS: phospho-endothelial nitric oxide synthase; ET-1: endothelin 1; IL-6: interleukin 6; CD36: cluster of differentiation 36; VSMC: vascular smooth muscle cells; ox-LDL: oxidized LDL; RAGE: receptor for advanced glycation end products; TNF: tumor necrosis factor; CCL 2/5/8: inflammatory chemokine ligands. This figure was created via BioRender (http://www.BioRender.com).

[47]. This finding is further supported by Gal-3-induced localization of the VEGF receptor and subsequent increase in VEGF signaling on endothelial cell membranes [48]. Animal studies revealed increased expression of Gal-3 by brain cells following ischemic injury with attenuation of postischemic angiogenesis following the inhibition of Gal-3 [49]. Knocking out Gal-3 gene has also proven to suppress VEGF upregulation following middle cerebral artery occlusion in a mouse model [50], which provides more evidence for VEGF involvement in postischemic angiogenesis induced by Gal-3.

In summary, Gal-3 not only is intimately involved in the atherosclerotic process but also contributes to postischemic

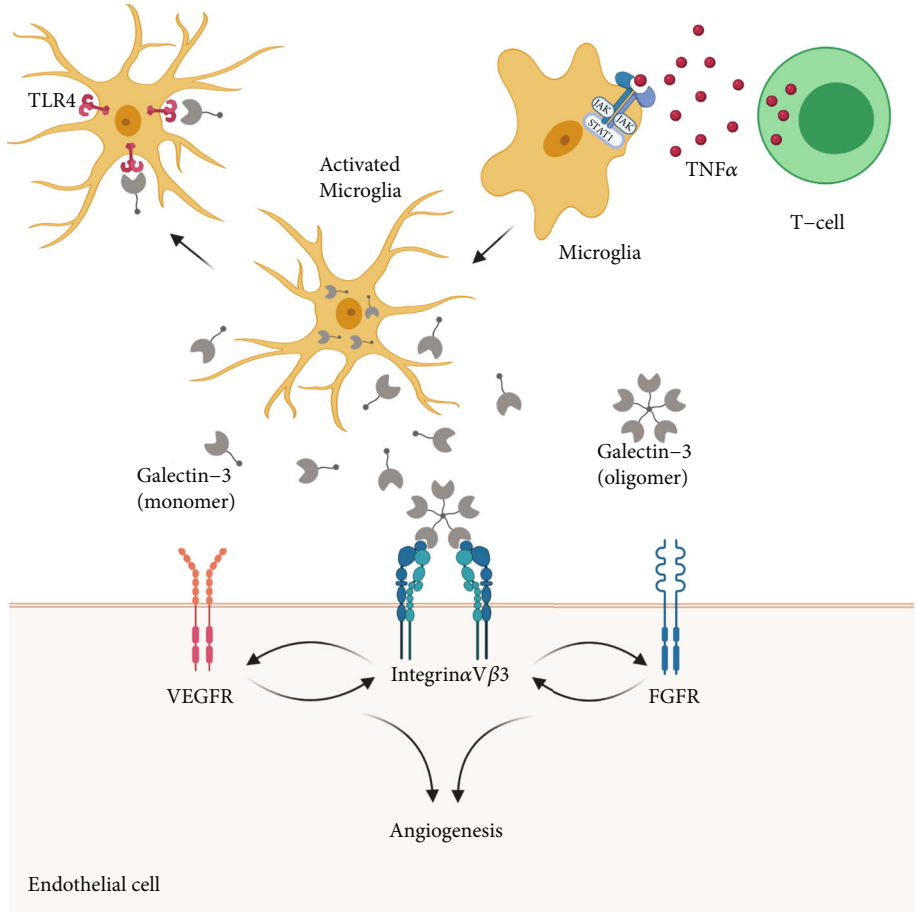

Figure 2: Potential effect of galectin-3 during stroke. TNFα: tumor necrosis factor; TLR4: Toll-like receptor 4; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor. This figure was created via BioRender (http://www.BioRender.com).

tissue remodeling by promoting angiogenesis and healing. It also has a fundamental role in regulating the immune response to acute brain injury. However, the answer to the questions of whether and how it acts to potentiate or to attenuate inflammation is still unclear. Its pleiotropic effects on the immune system coupled with its differential expression and the pathophysiological differences between different types of brain injuries suggest that our current knowledge is somewhat limited and that future studies need to take into account these variables. To that end, although the current lack of understanding of Gal-3's overall role in stroke limits its potential as a therapeutic target, it could still be a valuable biomarker to be used in the management of stroke patients, given that levels of Gal-3 increase significantly in response to acute brain injury.

4. The Role of Galectins in Stroke Prediction

4.1. Predictive Value in the General Population. Taking into consideration the vital role galectins were shown to play in atherosclerosis, a number of studies have investigated their utility in predicting the incidence of stroke in the general population, due to the well-known relationship between atherosclerosis and stroke. Jagodzinski and colleagues [31], via univariate analysis, initially showed a significant association between the levels of Gal-3 and stroke. However, this association became nonsignificant after adjustment using the Framingham risk factors in multivariate models, suggesting that it was Gal-3's positive association with typical stroke risk factors that drove the initial significant association, rather than a contribution independent of these risk factors. To evaluate the predictive utility of Gal-3, the investigators used three measures: the C-statistic, the integrated discrimination improvement (IDI), and the net reclassification improvement (NRI). Then, they assessed whether adding Gal-3 improved these predictive measures. Disappointingly, both the C-statistic and the IDI showed no significant improvements after adding the value of Gal-3. However, using the continuous NRI, they showed that Gal-3 addition resulted in significant improvements to the previous models, but the categorical NRI with four risk groups showed no such improvement [31].

Another study assessed the value of Gal-3 in improving stroke prediction in the general population through the analysis of the data from the Reasons for Geographic and Racial Differences in Stroke (REGARDS) cohort [29]. In this study, the significant association between Gal-3 and stroke events was no longer seen after adjustment for age, which encouraged the investigators to stratify the study participants by age, where a significant association was seen in the younger

(<64 years) but not the older study participants. However, it should be taken into consideration that even in the younger study population where Gal-3 was a more reliable stroke predictor, the association became nonsignificant after adjustment for Framingham risk and socioeconomic factors [29].

The investigators hypothesized that much of the association seen between stroke and galectins may have been due to galectin's association with intermediary inflammatory processes. In the elderly, these processes result into the development of diabetes and hypertension, both of which are disorders with well-known inflammatory components [51, 52]. However, in the younger population, inflammation may not yet have led to the development of hypertension and diabetes. The end result is that in younger populations, the effect of inflammation can only be accounted for by Gal-3, whereas in older populations, where hypertension and diabetes are already more prevalent, Gal-3's independent contribution becomes minimal. Indeed, this would also explain why Gal-3's association —even in the young— was also severely attenuated when adjusting for other typical stroke risk factors, such as hypertension and diabetes. This is consistent with the prior literature, where Gal-3 levels were reported to be significantly increased in diabetic patients as compared with prediabetics, suggesting a positive correlation between plasma glucose levels and the levels of Gal-3 [53]. This might be ultimately suggesting an association between Gal-3 levels and risk factors leading to stroke. An important limitation of the abovementioned studies that may have limited understanding the actual Gal-3's predictive value is that Gal-3 measurements were only done once at baseline and the investigators were not able to explore the value of changing Gal-3 levels and how they might impact future stroke risk.

All in all, Gal-3's predictive role may prove quite important in certain subsets of population, particularly younger individuals, whereas it may be relatively less useful in those with already-established risk factors for stroke. Further research should focus on confirming the utility of Gal-3 as an independent prognostic marker in younger individuals where it might be of most value.

4.2. Predictive Value in Populations at High Risk of Stroke. Studies aimed at assessing galectins' utility as a predictive tool for stroke have not been exclusively limited to the general population. Indeed, the utility of galectins in specific populations, known to be at particularly high risk of stroke, may be of a greater value than their utilization in the general population. Edsfeldt et al. [30] investigated the association between increased Gal-3 levels and stroke incidence following carotid endarterectomy (CEA) and found that even after adjusting for a number of important covariates (including demographic data, statin use, and smoking status), high Gal-3 levels maintained a significant association with stroke occurrence, with a hazard ratio (HR) of 4 (95% CI 1.6 to 10.4, $p = 0.004$).

Interestingly, subgroup analysis by gender showed that this association seemed considerably stronger in females (HR of 15.1, 95% CI 1.3 to 172.2, $p = 0.028$), as opposed to males, in whom the association was not significant. This may have critical clinical implications as the benefit of CEA in females has been a subject of debate [54], with a number of studies showing a higher risk of perioperative complications in females [55–57]. This debate is particularly pertinent in asymptomatic women with a lower grade (50-69%) of stenosis, in whom a meta-analysis of the Asymptomatic Carotid Surgery Trial (ACST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) demonstrated no significant benefit of CEA [55, 57, 58]. If females with the highest risk of stroke following CEA can be identified by measuring Gal-3 levels, this may hold much promise in terms of allowing physicians to selectively operate on those who are most likely to benefit from therapy. However, several considerations must be taken into account while looking at the abovementioned results. The results of the post hoc subgroup analysis by gender need further verification in the future studies as the reported confidence intervals for females were quite wide (1.3 to 172.2), which emphasizes the need for larger studies through which more precise estimates can be obtained. Moreover, the benefits of measuring Gal-3 levels need to be investigated in clinical trials evaluating whether implementation of routine Gal-3 measurements before CEA improves outcomes for patients in clinical practice.

Gal-3 has also been investigated as a predictive marker in dialysis patients, who are at a significantly higher risk of stroke than the general population due to the vascular alterations induced by uremia [59, 60]. In a cohort of dialysis patients, elevated Gal-3 levels were shown to be significantly associated with a higher risk of stroke [61], but after adjustment for confounding variables, Gal-3 only maintained its significance in relation to the composite endpoint of cardiovascular events including sudden cardiac death and nonfatal myocardial infarction as additional components rather than stroke.

5. Galectins as Predictors of Clinical Outcomes following Stroke

5.1. Postischemic Stroke. In light of its role in inflammation as well as resolution of acute brain injury, Gal-3 has become a biomarker of interest in the prediction of poststroke clinical outcomes. Both ischemic and haemorrhagic strokes are associated with neuronal damage and significant inflammation. Hence, in accordance with the in vitro studies, it is not surprising to find that Gal-3 levels are elevated in proportion to the severity of stroke [46, 62–64].

Ischemic stroke occurs as a result of decreased perfusion due to arterial stenosis/occlusion causing focal ischemia, hypotension, or increased intracranial pressure leading to global ischemia. Increased Gal-3 levels were reported to be positively correlated with stroke severity estimated by the National Institutes of Health Stroke Scale (NIHSS) score with an r-coefficient of 0.87 [46]. In addition, the receiver operating curve (ROC) analysis revealed that Gal-3 was able to predict the functional outcome of stroke at a cut-off value of 53.5, with a sensitivity of 88.4% and a specificity of 76.9% (area under the curve (AUC) = 0.884, 95% CI 0.827 to 0.941, $p < 0.001$), where functional outcomes were categorized into favourable and poor outcomes according to the modified

Table 2: Association of plasma Gal-3 levels with stroke outcomes.

Ischemic stroke					
Dong et al. [46]	Outcome	Sensitivity	Specificity	Cut-off value	
	Functional outcome	88.4%	76.9%	53.5 pg/mL	
	Outcome	Q1*	Q2*	Q3*	Q4*
Wang et al. [63]	Death or major disability (OR)	1.00	1.09	1.16	1.55
	Death (OR)	1.00	0.74	0.81	2.10
	Major disability (OR)	1.00	1.13	1.19	1.43
Haemorrhagic stroke					
Yan et al. ([64])	1-week mortality	73.3%	79.4%	28.9 ng/mL	
	6-month mortality	90.9%	64.6%	22.4 ng/mL	
	6-month unfavourable outcome	89.5%	65.5%	18.9 ng/mL	
Liu et al. ([62])	6-month mortality	77.8%	70.6%	24.6 ng/mL	
	6-month unfavourable outcome	81.1%	77.1%	23.4 ng/mL	

*Wang et al. categorized their study participants into 4 subgroups according to the quartiles of Gal-3 levels with Q4 being the highest quartile and Q1 being the lowest one. OR indicates odds ratio.

Rankin scale (mRS) (mRS score of 0-2 = favourable outcome; mRS score of 3-6 = poor outcome) [46]. In agreement with these findings, a multivariable adjusted-spline model showed a linear relationship between Gal-3 level and poor clinical outcome following stroke [63]. The authors also reported that Gal-3 was significantly associated with death and/or major disability 3 months postischemic stroke independently of other risk factors [63] (Table 2). On the other hand, a pilot study by Bustamante et al. [65] reported contradicting results suggesting that Gal-3 is not an effective biomarker for prognostic prediction in patients with ischemic stroke. However, this study had a very small sample size of only 26 patients, which limits the conclusion one could draw from this study's results.

5.2. Posthaemorrhagic Stroke. Haemorrhagic stroke can be classified into intracerebral and subarachnoid haemorrhage. Despite being less common than ischemic stroke, haemorrhagic stroke is associated with greater morbidity and mortality [66]. Therefore, being able to accurately predict the clinical prognosis following haemorrhagic stroke events could allow for modifying management plans according to the predicted clinical scenarios in order to achieve better outcomes. In a cluster of 110 patients with intracerebral haemorrhage, a multivariate analysis by Yan et al. [64] revealed that a high level of Gal-3 was an independent risk factor for a poor prognosis. Analysis of the ROC showed that the Gal-3 level was successfully used to predict one-week mortality, six-month mortality, and six-month poor outcomes with a moderate sensitivity/specificity at respective cut-off points (see Table 2). In the same cohort, Gal-3 was found to significantly improve the C-statistic of the mainstay predictors of the haemorrhagic stroke outcome (NIHSS score and hematoma volume), but this was only true for long-term outcomes (6-month mortality and poor outcome) [64]. Liu et al. [62] reported similar findings in patients with subarachnoid haemorrhage as elevated Gal-3 levels were an independent risk factor for six-month mortality and poor outcome with a sensitivity/specificity of 77.8%/70.6% and 81.1%/77.1%, respectively (Table 2). Nevertheless, there are discrepancies between the two studies. For example, Yan and colleagues [64] reported that Gal-3 levels were higher in nonsurvivors compared to survivors at 6 months, whereas Liu et al. [62] reported the opposite. It might be the difference in haemorrhagic stroke types between the two studies that is responsible for such discrepancies.

Overall, studies on the prognostic value of Gal-3 in the prediction of stroke outcomes are scarce, and the available data are limited by a number of factors. Firstly, most of the studies were done on relatively small population samples which makes it difficult to apply any of the findings to the general population of stroke patients. Furthermore, most studies showed that Gal-3 levels were only moderately predictive of the stroke outcome. As a result, it is difficult to make a definitive decision as to whether Gal-3 testing is beneficial as a prognostic marker for stroke. Based on that, more prognostic studies on larger population samples are needed.

5.3. Modification of Gal-3's Prognostic Effect by Glycaemic Level. Hyperglycaemia is a common presenting symptom of stroke that most likely occurs as a result of the release of cortisol and noradrenaline [67]. The relationship between hyperglycaemia and the efficiency of Gal-3 level, as a prognostic marker in stroke patients, was investigated. Zeng et al. [68] reported on the role of hyperglycaemia in improving the predictive function of Gal-3 levels. In a cohort of 3,082 ischemic stroke patients, hyperglycaemic patients with higher Gal-3 levels (≥10.58 ng/mL) were found to be at greater risk of developing the primary composite outcome (death and vascular events), stroke recurrence, and vascular events only one year after a primary stroke event, with an adjusted HR of 1.72, 2.64, and 2.68, respectively, as opposed to the normoglycaemic cohort in which Gal-3 had a low prognostic value [68]. The mechanism by which hyperglycaemia enhances the prognostic efficacy of Gal-3 is still unclear. Consequently, more molecular studies are needed to better outline the functions and interactions of Gal-3, and this in turn will allow improved clinical utilization of Gal-3 levels in special cohorts.

6. Conclusion and Future Recommendations

In summary, the current evidence suggests a potentially important role of galectins in the development and progression of stroke, and their interaction with other variables, such as plasma glucose level, requires further confirmatory studies. There are attempts to implement these findings in clinical practice by investigating the utility of galectins in two primary domains: the prediction of stroke occurrence and the prognosis of stroke.

As to the prediction of stroke, results seem to be promising when applied to specific subpopulations, such as younger individuals and female patients undergoing CEA. This suggests that galectins may have a greater diagnostic value if applied to specific portions of the population. Nevertheless, these results are in need of further confirmation by dedicated studies investigating the utility of galectins in those particular groups. Furthermore, future studies should also investigate the role of serial changes in Gal-3 levels and how they might impact stroke occurrence, as current studies have only investigated the effect of baseline measurements.

When it comes to the clinical prognosis of stroke, galectins have proven potential utility in both ischemic and haemorrhagic strokes, with studies showing an important association between the levels of galectins and clinical outcomes of stroke that is deserving of further study. Future studies integrating Gal-3 measurements in clinical settings may advance the management of patients with this debilitating condition.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Inflammation and Alzheimer's Disease: Mechanisms and Therapeutic Implications by Natural Products

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Alzheimer's disease (AD) is a neurodegenerative disorder with no clear causative event making the disease difficult to diagnose and treat. The pathological hallmarks of AD include amyloid plaques, neurofibrillary tangles, and widespread neuronal loss. Amyloid-beta has been extensively studied and targeted to develop an effective disease-modifying therapy, but the success rate in clinical practice is minimal. Recently, neuroinflammation has been focused on as the event in AD progression to be targeted for therapies. Various mechanistic pathways including cytokines and chemokines, complement system, oxidative stress, and cyclooxygenase pathways are linked to neuroinflammation in the AD brain. Many cells including microglia, astrocytes, and oligodendrocytes work together to protect the brain from injury. This review is focused to better understand the AD inflammatory and immunoregulatory processes to develop novel anti-inflammatory drugs to slow down the progression of AD.

1. Introduction

Alzheimer's disease (AD) is a slow, fatal, human neurodegenerative disorder depicted by the progressive loss in memory retrieval, learning, language, and other cognitive activities. Two main toxic misfolded protein fragments including amyloid plaques and neurofibrillary tangles begin to assemble inside the brain, which sequentially and moderately accelerates the advancement of the disease and eventually leads to neuronal dysfunction and cell death. Amyloid-β lesions and neurofibrillary tangles slowly obliterate the hippocampus, and forming new memories becomes more complex. Several etiological assumptions have been projected for AD, such as genetic abnormalities, the extent of neurofibrillary tangles, irregular APP processing, a discrepancy of neurotrophic factors, mitochondrial dysfunction, microelement neurotoxicity, impairment in energy metabolism, and oxidative stress [1]. AD leads to neuronal dysfunction and cognitive deficits by the accumulation of the amyloid-beta peptide (Aβ) in the human brain [2]. Short-term memory, visuospatial

dysfunction, and praxis are the most common symptoms of AD. Generally, inflammation is considered to be an intricate defense mechanism that appears internally in response to agitated and altered homeostasis. Neuroinflammation predominantly illustrates the inflammatory responses taking place in the central nervous system (CNS) which includes well-balanced innate and adaptive immune systems. Neuroinflammatory mechanisms immensely facilitate the development of the brain as well as in the neuropathological episodes. Cell-autonomous and non-cell-autonomous operations are the two separate pathological operations involved in neuronal death in AD as well as in most neurodegenerative diseases. The cell-autonomous procedure involves the occurrence of intrinsic impairment in the degenerated neurons which triggers their extinction, whereas the non-cell-autonomous operation involves the divergent deflation of the afflicted neurons stimulated by pathological interrelations with neighbouring cells, including astrocytes and microglial cells or immune cells such as macrophages and lymphocytes impregnated from the periphery.

The defensive mechanisms against various toxins, injury, and infection caused by several other means lead to the interruption in the expression of proinflammatory and anti-inflammatory cytokines and initiate chronic neuroinflammation [3, 4], which in turn triggers the liberation of various cytokines and the stimulation of microglial cells. Researchers postulated that continuous immune response is linked with neurodegeneration as well as the acceleration of both Aβ and NFT lesions and supports the insight into initial incidents of Aβ lesions followed by the progression of NFTs [5, 6]. Extensive studies have been carried out to distinguish the mechanisms involved in the progression and prevention of these two abnormal lesions of AD such as Aβ and NFT. However, there is no such treatment accessible that can effectively change both the pathologies involved in the development of AD [7]. Studies have investigated the response of inflammatory markers both in the postmortem and preclinical samples in AD patients, and they have suggested that neuroinflammation is the main and early feature of AD, which actively contributes to the pathogenesis of AD [8, 9]. In this review, we summarize the role and the mechanism of inflammation in AD and the therapeutic manipulation against the progression of AD.

2. The Baseline of Neuroinflammation in AD

Amyloid precursor protein (APP) is a glycosylated intermembrane protein, ubiquitously found in numerous tissues and abundantly found in the brain, that undergoes enzymatic cleavage and produces Aβ1-40 and/or Aβ1-42. APP functions as a trophic factor, which plays a substantial role in synaptogenesis, neurite outgrowth, and remodeling of synapses [10]. During neuronal differentiation and maturation as well as in the pathological events of AD, the expression of the APP protein gets subsequently increased [11]. The production of Aβ40-42 residues tends to aggregate into fibrils and deposit with age. Aβ1-42 is longer, hydrophobic, and fibrillogenic that is less produced than Aβ1-40. It is the principal species deposited in the brain [12, 13] and favors oligomerization and subsequent fibril formation [14, 15]. Aβ1-42 is deposited as the main component of senile plaques, and these oligomers are collectively produced by the activities of neurons and related astrocytes [16]. Aβ peptides are produced abundantly during the development of AD and start to accumulate in the brain. These oligomers subsequently destabilize microtubule-associated tau protein, which leads to its hyperphosphorylation and initiates its accumulation to form filaments in the neuron. Due to the destabilization of tau protein, the skeleton of neurons begins to degenerate and the communication between the neurons is lost [17]. It has been investigated in various studies whether senile plaques and NFTs are the only abnormalities involved in neuronal damage. Both imaging and postmortem studies showed that treatments for AD patients attenuated the Aβ pathology but could not alter its progression [18]. Thus, it has been suggested that apart from Aβ and NFTs, other factors are actively involved suggested that, apart from Aβ and NFTs, several other factors are also actively involved in the impairment of neurons in AD. Inflammation is the prime factor involved in the progression of various diseases including neurodegenerative diseases, which is validated by the increase in the expression of proinflammatory cytokines in the brain tissues and the blood samples of AD patients [19, 20]. Also, it gets expressed from the events of activated specialized macrophages such as microglia and astrocytes around the Aβ aggregates in AD [18].

Neuroinflammation also plays an active role in other neurodegenerative diseases, as indicated by the overexpression of proinflammatory markers, and is the target for therapeutic manipulations [21]. Several studies have proposed that inflammation triggers the increased accumulation of Aβ peptides, and the activated astrocytes and microglial cells are involved in the deposition of oligomeric peptides [22]. Several pro- and anti-inflammatory cytokines have been identified, in which proinflammatory cytokines are involved in the promotion of inflammatory responses, whereas anti-inflammatory cytokines trigger the regulatory responses to control the neuronal damage caused by proinflammatory cytokines. Several protein kinases such as glycogen synthase kinase 3β (NF-κB), mitogen-activated protein kinase (MAPK), cell division cycle 2 kinase (cdc2), and JAK-STAT are involved in AD progression. Thus, these kinases may be activated by elements of AD pathology such as inflammation, oxidative stress, Aβ, and cell cycle reentry [23]. Caspases are also known to play a significant role in inflammation; for example, caspase-1 plays an essential role in the maturation of proinflammatory cytokines, and other caspases such as caspase-3, caspase-7, and caspase-8 may regulate the activation of microglial cells [24]. Chemokines are also important factors that play an essential role as mediators in inflammatory processes. During inflammatory events in CNS, enhanced chemokines production and initiation of microglial and astrocyte chemotaxis takes place. Astrocytes and microglial cells perform significant activities in homeostasis and brain functions and are the main agents involved in the inflammatory processes in AD [25]. It is indicated that activation of microglial cells may be an early event in the pathogenesis of AD and may trigger the disruption of synaptic transmission and encourage early memory dysfunction [26]. In response to various toxins, astrocytes and microglial

cells get activated and lead to the process called reactive gliosis, which significantly contributes to the pathogenesis of neuroinflammation. In the reactive gliosis process, an astrocyte-specific intermediate filament protein (GFAP) plays a significant role in the homeostasis of CNS, and allograft inflammatory factor-1 (Alf1), a microglial specific protein, is found to be increased in an experimental study of AD [27]. The further inflammatory cascade is validated by the exaggeration of astrocytes and glial cells and the overexpression of proinflammatory cytokines in the AD brain [27]. Proinflammatory cytokines emphasize oxidative stress through the activation of NF-κB, and these transcription factors are the key players in the process of neurogenesis as well as in other physiological processes [28], and are involved in the process of synaptic plasticity [29]. Some assert that AD-affected brain regions contain enhanced levels of neuroinflammatory mediators via the increased inflammatory responses [30].

2.1. Effect on Cells of Inflammatory Response in Aged AD Brain. In different tissues, transcriptional variability in between cells including the hematopoietic cell lineage gets upregulated during aging [31, 32]. Also, a reduction in the population of naive T and B cells occurs, whereas terminally differentiated T cells increase in number in aged persons [33, 34]. Memory B cells are attenuated in aging, which leads to the reduced antibody response, while in aged humans and aged mice, mature B cells, termed age-associated B cells, may trigger autoimmunity and inflammation [35, 36]. In aging, an inefficient immune response makes the person more sensitive to various infections and autoimmune disorders, which are associated with and eventually leads to the deterioration in cognitive functions. During senescence, cells begin to assemble inside the body by accessing cell cycle arrest stimulated by stressors. It has been elucidated that an intricate mechanism is involved in the biosynthesis of these cells and in the significant role they play in various physiological and pathological events [37, 38]. The association of cells during senescence in host immunity is parallel to their potential to extrude proinflammatory cytokines known as senescence-associated secretory phenotype (SASP) [39]. NF-κB plays a significant role in the initiation of SASP which is triggered by various events such as stress, DNA damage, and developmental indications, which in turn promote the transcription of IL-6, IL-8, IL-1β, and TNF-α inflammatory markers [31]. Though microglia play an important role in the augmentation of neuroinflammatory responses and the early occurrence of Aβ oligomers, microglia begin to work against these toxic peptides by producing proteolytic enzymes that help to clear and destroy Aβ fibrils, initiate inflammatory responses, and assist in tissue homeostasis [40, 41]. In the normal functioning of the immune mechanism, activated microglia and the cytokines help to clear the foreign agents and rejuvenate the damaged tissues. The formation of Aβ continues during AD which leads neuroinflammation, which may instigate a vicious cycle that further leads to Aβ generation and alter the functioning of microglial cells [42, 43].

2.1.1. Microglia. Microglia, being the prime innate immune cells of the brain, play an indispensable role in the homeostasis of the CNS. They form and trigger the first line of defense inside the brain, thereby countering the pathological incidents through an array of inflammatory responses. Inflammatory cytokines, chemokines, and other markers are conferred to stimulate the activation of microglial cells, which potentially support injury and neuronal dysfunction. It is documented that APP, amyloid aggregates, and fibrils are potent glial activators, which stimulate an inflammatory cascade and the release of microglial neurotoxic cytokines [44]. Autopsies of AD brain patients exhibited reactive microglia adjacent to Aβ plaques; this has been explicated that Aβ triggers several pathways such as the NF-κB-dependent pathway, mitogen-activated protein kinase (MAPK) pathways, the cell surface binding of microglia, and the initiation of extracellular signal-regulated kinase to stimulate proinflammatory gene expression [25, 45]. The Aβ peptides are known to incite NADPH-intervened priming in microglia, which contributes to ROS generation that leads to neurotoxicity [46].

Several interleukins, including IL-2, IL-6, and IL-1β, tumor necrosis factor (TNF-α), and anti-inflammatory transforming growth factorβ1 (TGFβ1) were found in enhanced levels in AD subjects [25, 47], and the explication of the postmortem samples manifested the higher amounts of IL-1β, interferon-γ (IFN-γ), TNF-α, and NOS and the generation of free radicals. These observations further support the inclusion of microglial cells in the stimulation of an inflammatory cascade signifying the occurrence of several AD phenotypes and discrete function during the advancement of the disease. Several experimental investigations have been carried out on blood samples and cerebrospinal fluid (CSF) samples in AD patients, and these samples revealed enhanced expressions of proinflammatory markers including IL-2, IL-6, IL-1β, TNF-α, and IL-10 anti-inflammatory markers [48]. Probing of CSF has come up with new hope in providing essential information for the detection of unknown neurodegenerative and neuroinflammatory biomarkers, as it reflects more precise pathological and metabolic modifications in the CNS compared to other fluids.

2.1.2. Astrocytes. Besides microglia, astrocytes have also been found to participate in the synaptogenesis, neurotransmission, BBB constancy, and neuropathology of AD, which induces the expansion of proinflammatory cytokines [49]. These cytokines instigate secretases which support the Aβ generation from APP, and the reactive astrocytes contain BACE, an Aβ cleavage enzyme which plays a crucial role in Aβ production, and presenilin-1, which also favors Aβ production by releasing γ-secretase that cleaves the APP enzyme to give to Aβ oligomers. In the CNS, astrocytes not only play an indispensable role in Aβ production, but they also participate in the degradation of Aβ and form a protective barrier in between neurons and toxic peptides [50]. Astrocytes play an essential role in imparting neuronal development and synaptic plasticity in the CNS, energizing the neurons, and maintaining brain homeostasis. Elevated amounts of calcium-binding protein S100b are primarily represented by astrocytes which act as a cytokine, which has been reported in the postmortem studies of AD patients. Levels of inducible

nitric oxide synthase (iNOS) may get upregulated by S100b, resulting in the initiation of cyclooxygenase-2 (COX-2) in microglial cells and leading to the enhanced expansion of nitric oxide (NO) and superoxide radicals, which in turn may cause direct or indirect death of neurons. The C-terminal 100 amino acids of bAPP in AD pathology plays a crucial role in the instigation of astrogliosis and the death of neurons [47]. Microglia are involved in the phagocytosis and degradation of Aβ oligomers, while astrocytes play an essential role in the clearance and deterioration of these toxic peptides [51]. A study postulated that astrocytes in the cortex compile Aβ42 fragments which imperatively leads to the enhancement of Aβ pathologies, which confirms that astrocytes contribute to extended neuroinflammation, expressing iNOS that facilitates NO-induced toxicity [52]. Further, the appearance of active astrocytes in AD patients and AD animal models confirm the prominent role of these supporting cells of the brain in neurodegenerative diseases [51].

2.1.3. Oligodendrocytes. Oligodendrocytes are the crucial cells for neurotransmission and are the myelin-forming cells in the CNS. The oligodendrocytes together with the myelin sheath support the CNS and form an envelope around the axons, which act as an insulator and initiate smooth transmission of signals in between neurons [53, 54]. Studies manifest that improper functioning of oligodendrocytes may trigger the pathophysiology of diseases such as bipolar disorder, schizophrenia, AD, PD, and several other neurodegenerative disorders [55–58]. Several studies reported that pathological lesions and demyelination in AD white matter and Aβ fibrils in the grey matter have been substantially manifested in sporadic, familial, and mouse models of AD [59, 60]. Oligodendrocytes are involved in the modulation of ion homeostasis, and oligodendrocyte precursor cells (OPCs) are the specific cells that take part in the formation of synapses with glutamate neurons in the cortex and hippocampus, as well as in other areas of the brain [61–63]. We could speculate that neuronal AD pathology, which is characterized by neuronal and axonal dysfunction, could alter the amounts of myelin produced by oligodendrocytes. Thus, this dynamicity of myelin and oligodendrocytes could ultimately affect behavior. Several studies demonstrate that Aβ oligomers are involved in the process of demyelination and the loss of oligodendrocytes and having less antioxidant and iron content; these cells are more susceptible to oxidative stress, which is one of the main protagonists in inflammation [64, 65]. Also, inflammation is associated with activated oligodendrocytes in AD conditions [66]. Thus, inflammation, myelination, and demyelination are closely associated with oligodendrocytes (Figure 1).

2.2. Inflammatory Response Pathway Mechanisms in AD and Their Potential Therapeutic Targets. Composing the exact descriptions of AD is still underway, and continuous efforts are being made to reduce the progression of the disease. Presently, there are no treatments available to control cognitive impairment. Excessive production and the accumulation of Aβ-amyloid and hyperphosphorylated tau protein are the two main events that are considered the principal cause of AD. However, excessive Aβ generation may subsequently lead to immune dysfunction rather than the advancement of the disease itself. Given this, several pathways such as the NF-κB, MAPK, and JAK-STAT pathways are engaged in innate immunity and are linked with the expansion in AD [67, 68]. Several studies have investigated the possible mechanisms of these pathways and targeting therapeutic drugs for the inflammatory conditions in AD, which provide promising results against AD. Efforts are constantly being implemented to comprehend the AD pathophysiology and the involvement of several kinase pathways in the process of memory impairment and cognitive dysfunction. Therefore, in the following segments, we will emphasize the role of NF-κB, MAPK, and JAK-STAT pathways in support of the mechanisms associated with AD pathology.

2.2.1. NF-κB Pathway. Activation of microglial cells results in the formation and release of proinflammatory cytokines, which trigger neighbouring astrocyte neurons to generate further production of $A\beta_{1\text{-}42}$ oligomers, which may promote the upregulation of neuronal cell death by further activating inflammation [16]. Several therapeutic drugs are being used to inhibit neuroinflammation linked with disease progression, which reveals positive effects through various mechanisms such as maintaining Ca^{2+} homeostasis, inhibition of cyclooxygenase (COX), and targeting γ-secretase [69]. Several studies reported that toxic agents were found to be involved in the activation of regulatory inflammatory marker NF-κB by triggering the degradation of inhibitor kappa B (IκB) [70]. Overexpression of TNF-α plays an essential role in the activation of transcription factor NF-κB [71]. Proinflammatory cytokines emphasize oxidative stress by provoking expression of the iNOS gene via activation of NF-κB. The NF-κB factors play a key role in the CNS in several physiological processes associated to signal transmission, cognition, and memory [29]. Dysfunction of the CNS, oxidative stress, and neuroinflammation are the major events in AD which are activated and progress via activation of NF-κB. Generation of ROS triggers the IKKb enzyme which phosphorylates the heterodimer of NF-κB, an inhibitor of kappa B (IκB) causes its degradation by the ubiquitin-proteasome pathway, and the detachment of IκB from the dimer initiates the influx of NF-κB into the nucleus.

NF-κB acts as a prime factor of innate immunity in environmental and genetic risk factors in different AD models [72]. NF-κB signaling promotes BACE1 gene expression and Aβ processing and is considered to be the novel mechanisms essential for the advancement of AD [72]. Also, it has been reported by several studies that Aβ induces the enhanced expression of NF-κB which in turn facilitates the expression of chemokines and cytokines, and these proinflammatory markers promote the progression of AD. In addition, activation of NF-κB plays a crucial role in upregulating the levels of various microRNAs in the brain which further causes the diminishing of regulating proteins including synapsin-2, tetraspanin-12, and complement factor H in the CNS. Diminished extents of synapsin-2 lead to the dysfunction in synaptogenesis inside the neurons, whereas the declined proportion of tetraspanin-12 and complement

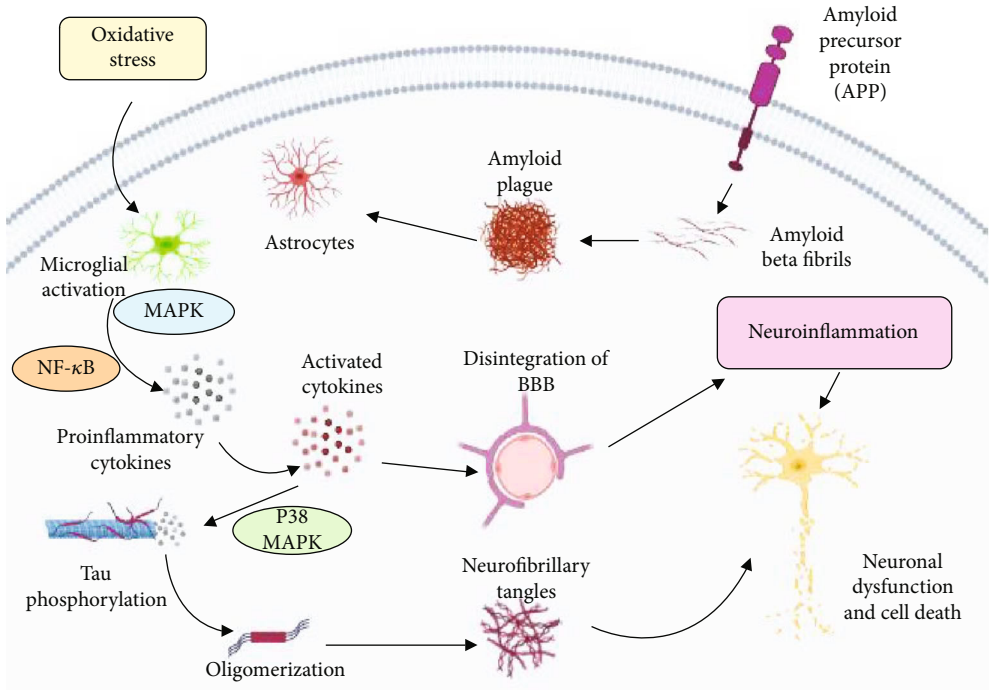

Figure 1: Aβ fibrils lead to neuronal death, which include ROS generation, neurotoxicity, release of inflammatory cytokines, and activation of the complement system. Due to the accumulation of Aβ oligomers, neuronal degeneration may stimulate the microglial activation, which will initiate the liberation of proinflammatory mediators, neurotoxins, and free radicals but also play a pivotal role in the elimination of Aβ peptides. These peptides trigger oxidative stress and promote inflammatory processes in neurons, which enhance the production of Aβ peptides via increased APP expression. Activated MAPK (a mitogen-activated protein kinase) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) lead to the production of proinflammatory cytokines, and their increased expansion promotes APP processing and disintegration of BBB (blood-brain barrier) and aggravates the phosphorylation of Tau protein and eventually leads to the formation of neurofibrillary tangles via the activation of p38-MAPK which leads to neuronal degeneration (created with http://BioRender.com/).

factor H promote Aβ accumulation and initiate an inflammatory response in the neuronal cells [73]. Therefore, several neurological diseases are pathologically associated with the activation of NF-κB. The NF-κB pathway is supposed to play a significant role in regulating the cellular fate in a broad array of physiological and pathological conditions, which provides an opening to exploit its essential functions. Several other factors such as phosphorylation and degradation of IκB, DNA interaction, and its translocation are found to be associated with the activation of NF-κB, which supports its compatibility for drug intervention. Numerous therapeutic agents have been implicated and have exhibited promising results in modulating the activation of NF-κB in the CNS and attenuating the processes which initiate the decline of neurons. Polyphenols, antioxidants, and several other drug categories have been used and are usually preferred as these therapeutic agents claim specific targets.

Antioxidants, polyphenols, and nonsteroidal anti-inflammatory drugs (NSAIDs) have been found to inhibit the activation of NF-κB and have played an instrumental role in attenuating the Aβ burden [74]. Curcumin, a polyphenolic compound, has been widely studied and has been proven to be a potential therapeutic and neuroprotective agent which significantly attenuated the neuronal death and alterations in brain tissue by regulating the NF-κB pathway [75]. Several studies reported that plant-derived compounds significantly downregulated the expression of NF-κB and mitigated the neuroinflammation in cellular models of AD [76, 77]. Immense efforts have been instigated in understanding the mechanism of the progression of the disease and the efficacy of novel therapeutic agents for AD. Several therapeutic approaches have been employed to regulate the disease progression and attenuate the cognitive deficit in AD subjects. Researchers have recently proposed that decreasing the expression of genes associated with AD may play an essential role in diminishing toxicity caused by misfolded Aβ and Tau proteins which may attenuate the advancement of AD [78]. siRNA gene silencing is one of the therapeutic approaches employed in AD subjects to attenuate disease progression. Several studies have revealed that siRNA silencing may play an essential role in regulating the expression of genes and in silencing AD-associated genes such as APP, Tau, and BACE1 genes. Thus, it exhibited positive results in diminishing mRNA levels of target genes, which confirms the efficacious applications of the siRNA approach in AD. [79–81]. Several other studies also confirmed that the APP gene silencing in the transgenic mouse model may attenuate the Aβ extents and may significantly improve the cognitive dysfunction in mice, which further confirms the advancing role of siRNA gene silencing and is considered a potential therapeutic approach for AD pathology [82, 83].

2.2.2. MAPK Pathway. These protein kinases are intracellular enzymes that make them feasible for cells to respond from the external environment; for example, inflammatory cytokines or osmotic shock triggers GTPase-dependent activation of several kinases. These kinases are involved in the phosphorylation and activation of MAPK kinases, which further phosphorylates and activates p38 MAPK and results in various adaptive responses. The p38 MAPK activation initiates the enhanced production of cytokines via direct phosphorylation of transcription factors and enhanced mRNA translation which code for proinflammatory cytokines [84]. In AD, postmortem studies have reported that exaggerated immunoreactivity of phospho-p38 MAPK was linked with Aβ plaques and neurofibrillary tangle-oriented neurons, and it was further observed in patients with AD that activation of p38 MAPK begins at the early stage of the disease [85, 86]. The upstream activator of p38 MAPK includes MKK6 that is in higher levels in postmortem brains of AD, which exhibits an active role of p38 MAPK in the advancement of the disease [67]. Activation of MAPK is the crucial factor for the inflammation event in the brain and has been investigated in several in vitro studies. A study showed that p38 MAPK leads to the activation of glial cells, in which both p38 MAPK and Erk cascades play an indispensable role in the transcriptional and posttranslational regulation of iNOS and TNF-α gene expression in activated glial cells [87]. The MAPK pathway has been found associated with amyloid-β fibrils and directs the transcription of inflammatory markers. Amyloid-β fibrils in microglia trigger brief and quick p38 MAPK activation, which results in the expression of inflammatory genes and enhances expression of proinflammatory cytokines [88]. In addition, it has been documented that microglial activation by APP mostly occurs via the p38 MAPK pathway, which initiates the generation of proinflammatory markers [89], and also it has been observed in an animal study that microglial MAPK signaling is involved in Aβ-induced neuroinflammation [90]. However, the p38 MAPK signaling pathway also plays a major role in the detrimental functions of activated astrocytes. Experimental data confirms that the release of IL-1β from microglia affects astrocytes, which leads to the activation of the NF-κB pathway [91], and IL-1β has been found to play a role in the activation of the MAPK pathway both in animal and human astrocytes [87, 92]. Though p38 MAPK leads to the production of iNOS and TNF-α, which causes a chronic inflammatory event, the regulatory function of the p38 MAPK cascade has been validated via the IL-1β-dependent inflammatory aggravation navigated by primary astrocytes. Also, it has been confirmed that regulating the activity of p38 MAPK causes the obliteration of the transcriptional activity of NF-κB and diminishes the expression of the NF-κB-dependent gene iNOS [93]. Several observations have shown new features in the p38 MAPK cascade are associated with AD pathology and extend all the way to designating p38 MAPK pathway inhibitors as therapeutic targets for the prevention of AD pathologies. Extracellular Aβ aggregates initiate and enhance MAKK6 which phosphorylates p38, which in turn initiates tau hyperphosphorylation, proceeds to neuronal apoptosis, and regulates synaptic plasticity. All these events are associated with the activation of p38 MAPK. Neprilysin, a cleavage enzyme involved in the degradation of Aβ, is also known to be afflicted by activation of p38. A study showed that SXFAD mice deficient in p38α, and the knockdown of p38α, leads to enhanced Aβ degradation by increasing the expression of neprilysin, a metalloproteinase in glial cells [94]. The activating transcription factor 4 (ATF4) is another factor that enables the activation of proapoptotic proteins of p38 by initiating MKK6 [95], and p38 helps in binding the Atf4 factor to the CHOP promoter and proceeds in the expression of caspase-4 [96].

Several experimental studies have investigated the therapeutic efficacy of p38 MAPK as a drug target which manifested the role of p38 MAPK inhibitors against neuroinflammation. Pinocembrin, a natural flavonoid, has been used in a study that attenuated the cerebral cortex neuronal degeneration and ameliorated the memory and cognitive function in the AD mouse model. It also showed remarkable results in diminishing the p38 MAPK activation and NF-κB signaling [97]. Another study reported the efficacy of an essential oil Z-ligustilide (Z-LIG) extracted from umbelliferous plants, which showed reduced levels of intracellular ROS and regulated the apoptosis in Aβ-induced toxicity in PC12 cells [98]. Moreover, it manifested promising results in protecting against neurotoxicity via the activation of PI3K/Akt and the regulation of the p38 MAPK pathway in differentiated PC12 and SHSY5Y neuroblastoma cells [98, 99].

2.2.3. JAK-STAT Pathway. The signal transducer and activator of transcription (STAT) factors are provoked by several cytokines and growth factors [100], and the disease and age-dependent decline in the JAK2/STAT3 pathway plays a central part in the AD pathogenesis. These growth factors are involved in the modulation of several genes, caspases, and the cell cycle regulators, which can also counter oxidative stress [100], and these factors are phosphorylated at tyrosine residues in reaction to several cytokines and growth factors [101]. Phosphorylation of STAT leads to its dimerization, and it becomes capable of binding to DNA, whereas in an unphosphorylated state, STATs are located in the cytoplasm and transfer to the nucleus on activation [102]. For the treatment with interferons, the Janus kinase (JAK) is essential for the phosphorylation of STAT factors [100]. The STAT members are diverse in their function and are accounted to play a crucial role in differentiation, proliferation, and cell survival [90–103]. They function as essential mediators in cytokine receptor signaling and are involved in the modulation of transcriptional target genes. It has been investigated that inactivation of STAT3 is associated with the AD pathogenesis, and the specific antibody has been analyzed against the phosphorylated form of STAT3, which exhibited its reduced levels in hippocampal neurons in AD patients [104]. In the context of higher levels of Aβ soluble peptides observed both in sporadic and familial forms of AD, it is postulated that these toxic peptides are involved in the inhibition of STAT3 proteins. In addition, several other pieces of evidence support that activated STAT3 was found to be attenuated in hippocampal neurons in AD mice models [105], which proposes that Aβ levels are inversely proportional to the levels of

activated STAT3 in the hippocampal neurons. These toxic peptides and STAT3 are having direct effects leading to the alteration of STAT3, which was confirmed from the in vitro study that Aβ induction persistently attenuated the STAT3 levels in primary hippocampal neurons [104]. Aging is also considered one of the factors leading to the inhibition of STAT3 in association with AD, as observed from the study that immunoreactivity of p-STAT3 in hippocampal neurons was found to be upregulated compared to the older subjects in animal and human models [104].

The JAK-STAT pathway acts as a possible therapeutic target to the toxicity, as it is involved in the microglial activation and the modulation of inflammatory responses [106, 107]. Cytokines and the ligand binding to their respective receptors enhance the action of tyrosine kinase of JAK, which leads to the activation of receptors by phosphorylating them and triggering STATs and their phosphorylation as well and its dimerization successively leads to the nuclear translocation and transcription function. Previously it has been documented that the JAK-STAT pathway is linked with inflammatory activities and has been targeted for therapeutic compounds, and it is under clinical trial [108]. Large amounts of Aβ contents are associated with the enhanced phosphorylation of tyrosine in animal and human models [109], whereas the JAK-STAT3 pathway regulation manifested the inactivation of microglia and astrocytes in animal models of AD [110].

Phytochemicals are being used extensively and have been implemented in the treatment of various disorders. They exhibit efficient therapeutic results against the diseases which gained the interest of scientists towards the plant-derived compounds and can target several pathways in the liberation of their therapeutic effects. Curcumin a natural flavonoid has been used in an experimental study that manifested the anti-inflammatory, antioxidant, cardioprotective, and neuroprotective effects [111–114]. Curcumin has shown promising results against various neurological diseases and proved to be effective in attenuating memory dysfunction and improved cognitive function [115]. Inflammation plays an influential role in the arrival of several disorders, which can be regulated by targeting the JAK-STAT signaling pathway [116]. Its inhibition shows positive results in diminishing the inflammation via attenuating the levels of inflammatory mediators [117]. Several phytochemicals such as curcumin, resveratrol, and epigallocatechin, etc manifest promising therapeutic results and anti-inflammatory response by attenuating the levels of inflammatory markers via targeting the JAK-STAT pathway [118–120] (Figure 2).

2.3. Other Mechanisms Driving Neuroinflammation and Their Potential Targets

2.3.1. Activation of Oxidative Stress Pathway (Redox Signaling). Oxidative stress is one of the factors responsible for the consequences of neuroinflammation. It is the mechanistic factor in the progression of inflammatory responses. Hydrogen peroxide and nitric oxide oxidants can come into existence either internally by initiating signal transduction processes or can get into the cell from the external environment. Cytokines are involved in the production of ROS via NADH oxidase (NOX) which is a ubiquitous mechanism commonly intimated for inflammatory factor-stimulated intracellular ROS production. The reduction of oxygen is catalyzed by NADPH oxidase to produce superoxide anion and is being released either at the surface of the cell or inside the cellular compartments [121]. Inducible nitric oxide synthase (iNOS) together with the microglia and astrocytes trigger the generation of nitric oxides. ROS/RNS perform significantly upon NO, thiols, and redox sensors. Cellular thiols react to produce S-nitrosothiols (RSNO) and sulfenic acids (RSOH), and both these contents undergo exchange with GSH to yield protein SSG species [122, 123], which is reprocessed to its active form. Glutathionylation is one regular content in redox signaling and ROS/RNS are also other mechanisms involved in redox signaling reacting with heme and transcriptional factors [123, 124]. The outcome of ROS/RNS initiated redox signaling in the neuroinflammatory motif is generally an increased expression of transcription factors that regulate the levels of chemokines, cytokines, metabolizing catalases, and ROS-producing enzymes. Cytokines are involved in the stimulation of iNOS in astroglia and microglial cells by directly venturing surface receptors leading to the generation of increased NO that may be toxic and become a threat to neurons. It has been investigated that AD brains exhibited higher production of iNOS and knockout of iNOS genetically has been observed to be protective against AD in mouse models [125]. Additionally, elevated levels of iNOS in AD have also been exhibited to be associated with NO-induced posttranslational modifications such as S-nitrosylation, nitration, and dityrosine generation [126]. At the tyrosine 10 residue, nitration of the Aβ peptide manifested the tendency of Aβ to accumulate and be recognized in the senile plaques [127].

2.3.2. Activation of Complement System Pathways. The complement system is the principal component and indicates a fundamental pathway for the activation of immune reactions and provides defense against foreign harmful agents. This system is also activated in neurodegenerative disorders and the proteins included are linked with pathogenic lesions in AD. These complement proteins once activated lead to the opsonization and support the proteolysis of microorganisms. Astrocytes do take part in the production process but to a little extent, whereas the microglial cells generously lead the production of proteins of the complement system [128]. A study reported that functional complement proteins are linked with Aβ toxic deposits and also the Aβ peptides are playing a critical role in the activation of the complement system observed in in vitro studies through the alternative pathway [129]. Further, it was confirmed from the study that Clustrin a heterodimeric protein (apolipoprotein J), acts as a soluble inhibitor and the complement receptor-1 is involved in the progressing and removing of opsonized immune networks linked with AD, proclaiming the significance of the complement system in AD [130, 131].

2.3.3. Activation of Cytokine and Chemokine Pathways. Cytokines are the key players that include proinflammatory and

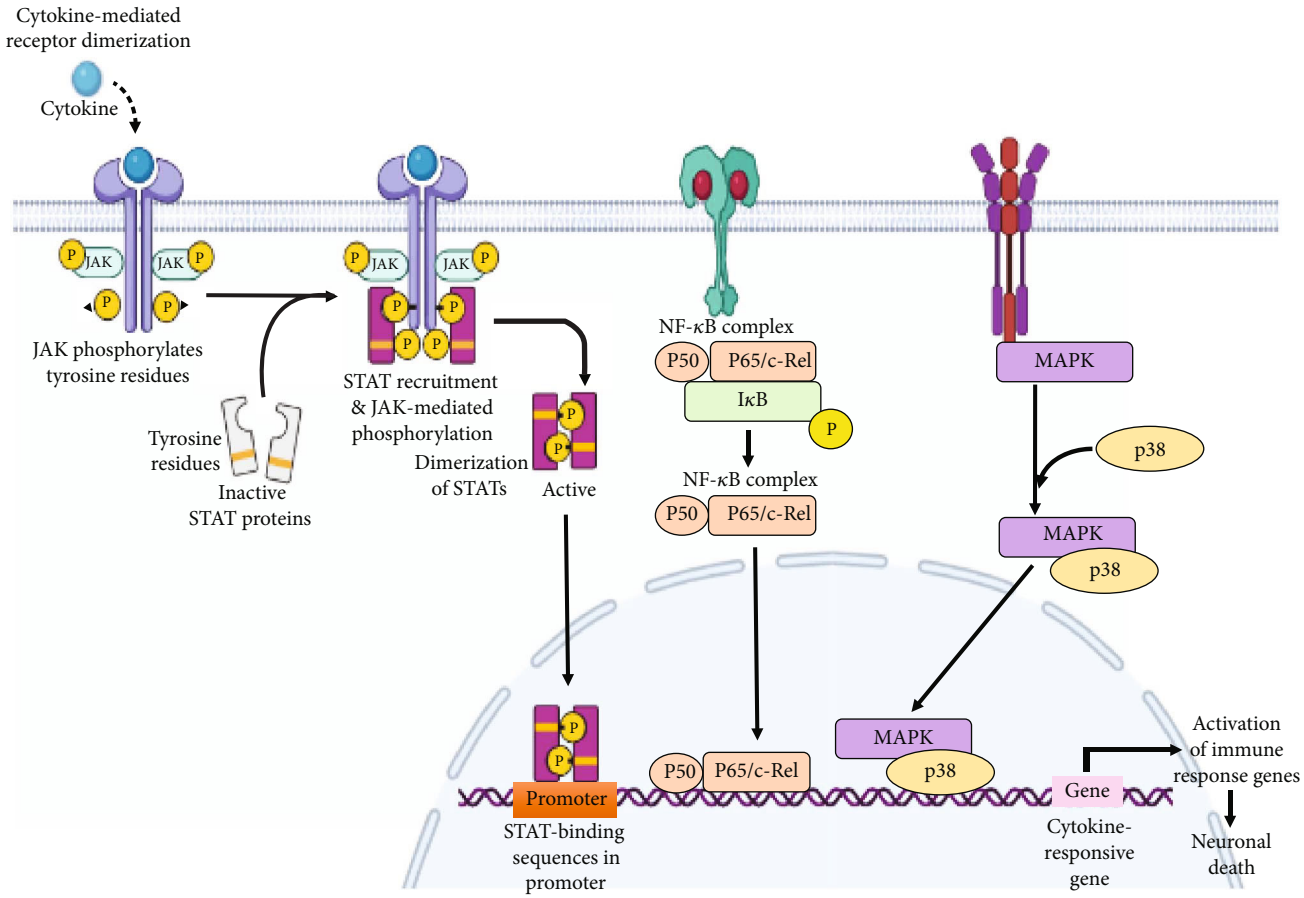

Figure 2: Inflammatory cytokines initiate the activation of the PI3K (phosphoinositide 3 kinase) pathway, phosphorylate the JAK-STAT (Janus kinases, signal transducer and activator of transcription proteins) factors, which activate the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway and enhance the production of ROS leading to apoptosis. p38 MAPK (a mitogen-activated protein kinase) is involved in the AD mechanism which includes the cytokine activation of p38 MAPK in microglia, leads to the increased production of proinflammatory cytokines which initiates the inflammatory process, and the cytokines also initiate the activation of p28 MAPK in astrocytes and neurons, which further escalates the inflammation. All these events lead to hyperphosphorylation, inhibition of long-term potentiation, apoptosis and synaptic dysfunction. NF-κB is a regulated transcription factor, involved in the regulation of inflammation, cellular growth, immune function and apoptosis. Free radical production led to the activation of IKB which phosphorylates the NFκB inhibitor, initiates the proteasomal degradation of IKB (IkappaB kinase) and the liberation of NFκB which translocates into the nucleus and binds to the DNA responsive element. Together with the coadjuvant and other activators, the increased expression of proinflammatory cytokines is triggered and neuroinflammation is supported, which causes the degeneration of neurons and eventually leads to the progression of AD (created with http://BioRender.com/).

anti-inflammatory cytokines involved in the neuroinflammation process and are produced by both astrocytes and microglial cells. A study revealed that in aged transgenic mice, expansion of Aβ was observed which is associated with the upregulation of proinflammatory cytokines [132]. This study supports that Aβ is the key player that triggers neuroinflammatory responses in AD. Exposure of microglia to Aβ promotes the activation of proinflammatory cytokines and macrophage colony-stimulating factor (M-CSF) [133]. In the CNS and plasma of AD patients, contents of M-CSF were depicted in an elevated state compared to healthy subjects [134, 135]. A subsequent higher level of IL-1β was observed in AD patients, and the activation of caspase-1 is essential for the activation of IL-1β, which has been found in an increased state in MCI and AD patients [136]. TNF-α is considered to be the most critical proinflammatory factor in AD, which promotes and modulates the cytokine members during the process of inflammation [137]. It can expand the endothelial adhesion proteins and directs the immune cells to get recruited in the defective area [138]. It is confirmed from the study that in the plasma and brain tissues of Also, TNF-α can play an instrumental role in enhancing the Aβ elements via the enhanced production of β-secretase and advanced activity of the γ-secretase enzyme [139, 140]. Chemokines are involved in the regulation of microglial movement to the affected areas by the process of neuroinflammation in AD, associated with the development of CNS, and are communication factors in between the cells [141]. The CCL2 chemokine and its receptor play a critical part in controlling the crossing over of peripheral monocytes inside the brain, which confirms its purpose in AD [141]. There is an increase in the activity of chemokine CCL2 and

the receptors CCR3 and CCR5 in reactive microglial cells, and there is a depiction of chemokine CCL4 in astrocytes around Aβ plaques [142]. It has been demonstrated that the induction of Aβ upregulated the levels of CXCL8, CCL2, and CCL3 while assessing the microglial cultures procured from the autopsies of AD [143]. Moreover, CCR2 and CCR5 receptors have potential to attenuate the advancement of the disease by impacting the function of microglial cells [144, 145].

2.3.4. Activation of Cyclooxygenase Pathways. Cyclooxygenases (COX) are involved in proinflammatory responses, and their expression is upregulated when inflamed and provoked in cells and tissues, which play a significant role in the generation of prostanoids [146]. It helps in the conversion of arachidonic acid into prostaglandinH2 (PGH2), where the prostanoids begin to synthesize with varied functions. COX-2 acts as a regulating factor in moderating the synaptic transmission localized at the post-synaptic sites but it has been documented that overexpression during pathological conditions may trigger neuronal damage and cognitive dysfunction [147]. Moreover, the excessive activation of COX-2 in neurons may trigger the expansion of Aβ generation and cognitive impairment has been investigated from various mouse models [148, 149]. This finding supports the activities of COX-2 and its outcomes in AD.

2.3.5. Opening of Blood-Brain Barrier with Aging. The blood-brain barrier (BBB) functions as a main defensive factor in the periphery of the brain which restricts the unspecific elements to make entry into the brain and inhibits the passaging of cells and molecules in between blood and brain tissues. During AD events, BBB becomes deficient and loses the selective and defensive function, as noticed from the inflammatory responses in the CNS at the site of injury and systemic infection leads to the disintegration in dementia [150, 151]. However, in normal conditions, BBB efficiently passes the Aβ into the blood and restricts the entry of Aβ from the serum into the CNS, whereas all these normal events are manipulated and disrupted during the progression of AD. Aβ42 peptide is involved in the alteration of the tight junction among endothelial cells of the BBB, making them deficient and punctured which simply causes the accumulation of Aβ fibrils and produces further burden and toxicity to the BBB. Moreover, proinflammatory cytokines such as TNF-α and IL-1β also take part in the distraction of tight junctions which are produced in excessive quantity by microglia in AD conditions [152], which makes the easy entry of macrophages and T cells in the CNS. Thus, these additional cells cause the extra alteration of astrocytes and microglial cells to initiate the inflammation, which leads to the additional liberation of inflammatory markers (Figure 3).

3. Role of Inflammation on Tau Pathology in AD

Inflammation is explicated as a serious event and plays an instrumental role in tau pathology. Around the toxic lesion in the brain, the appearance of activated astrocytes and microglial cells, and the enhanced expression of inflammatory markers indicate the interrelation between the AD pathology and inflammation [47]. A study was conducted on P301S mutant tau transgenic mice which manifested that the microgliosis and synaptic pathology may be the early events of neuronal dysfunction linked to tauopathy [153]. Excessive activation of microglial cells may play a role in the formation of tangles, while the attenuated tau pathology has been observed in the transgenic mice via immunosuppression, which highlights that neuroinflammation is the key event in the advancement of tau pathology [153]. There are several means such as physical or chemical injury or infections caused by pathogens, which trigger inflammation in the brain regions and leads to the direct involvement and activation of astrocytes, microglial cells, and inflammatory cytokines. Inflammatory processes and oxidative stress in AD lead to the activation of several kinases including MAPK, GSK-3β, and cdc2 [23]. An experimental study revealed that chronic induction of LPS subsequently triggered the phosphorylation of tau protein at various sites related to tau pathology in transgenic mice [154]. Also, the stimulation of microglial cells and IL-1β liberation were engaged via the CDK-5 or GSK-3β kinase activation [155]. The persistent activation of TNF-α has also been found to be associated with the formation and increase in the production of pre-tangle-linked pT231 epitope [156]. Proinflammatory cytokine interferon-γ (IFN-γ) plays a crucial role against viral infections, however, the increased expression of this cytokine was observed in a study that leads to the dephosphorylation of tau protein at phosphorylation sites [157]. Thus, confirms that the activation or inhibition of tau pathologies are linked to the proinflammatory cytokines and the activated glial cells (Figure 4).

3.1. Activation of Inflammasome. The number of pattern recognition receptors (PRRs) are provoked by various cell lineages inside the blood to detect and fight against pathogens [158]. PRPs are classified into two types based on their intracellular localization including Toll-like receptors (TLRs) and c-type lectin receptors (CLRs) which are localized in the cellular membrane and endosomes where they identify extracellular pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). A subset of PRPs contains NOD- (nucleotide oligomerization domain-) like receptor (NLR) and AIM2- (absent in melanoma 2-) like receptor (ALR) proteins, which can congregate multimolecular intracellular complexes called inflammasomes, which in turn can generate potent inflammatory reactions in response to stress and cellular contaminations [158, 159]. In an ATP-dependent manner, oligomerization of the NOD domain promotes activation of the inflammasome. The inflammasome contains nucleotide-binding and oligomerization domains and a precursor of procaspase-1 [158].

Several inflammasomes have been recognized such as NLRP1, NLRP3, IPAF, and AIM2, among them, NLRP3 is the most extensively studied inflammasome comprised of the ASC adaptor, NLRP3 scaffold, and procaspase-1 [158]. This inflammasome participates in metabolic as well as inflammatory disorders including diabetes and

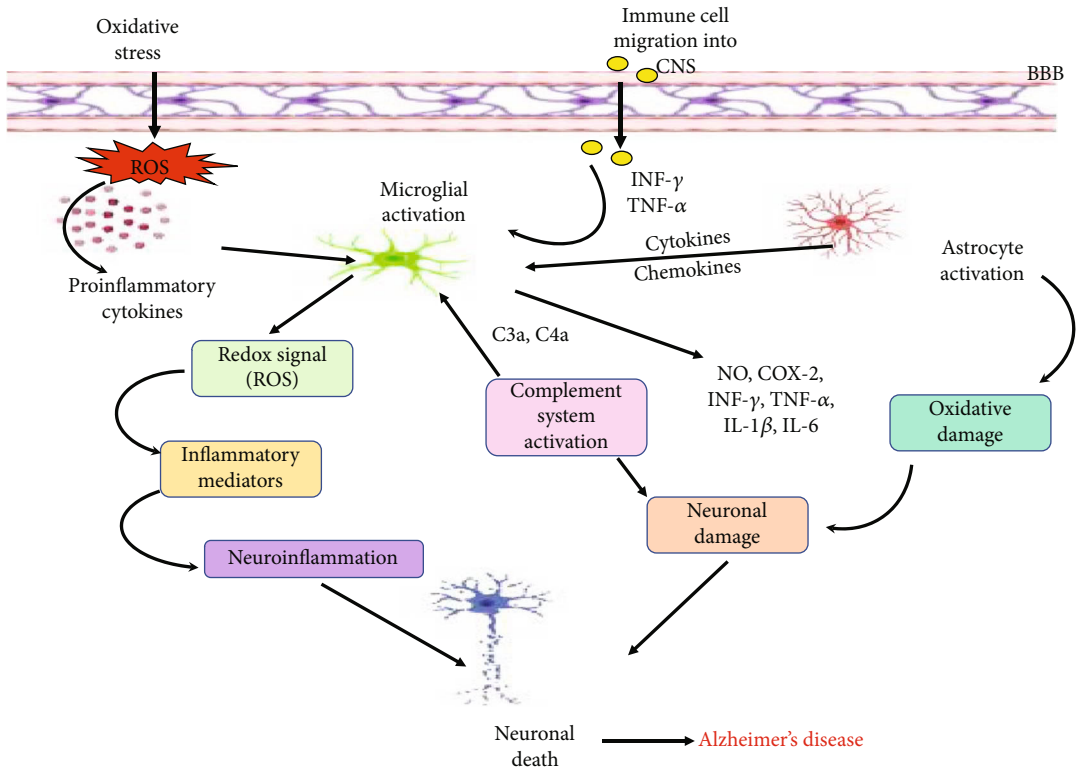

Figure 3: Other mechanisms driving neuroinflammation: increased oxidative stress either by excessive production and release of ROS (reactive oxygen species) of inflammatory mediators leading to the overproduction proinflammatory cytokines. Proinflammatory factors activate the glial cells and promote the process of neuroinflammation. Several antioxidants including SOD (superoxide dismutase), Cat (catalase), and GPx (glutathione peroxidase) may act as reducing agents in attenuating ROS production and diminish the inflammatory response. Activated glial cells under the influence of several proinflammatory cytokines trigger the complement system, and the released cytokines form T cells. Activated glial cells further promote the release and activation of inflammatory cytokines such as TNF-α (tumor necrosis factor), IL-1β (interleukin-1β), IL-6 (interleukin-6), NO (nitric oxide), COX-2 (cyclooxygenase-2), IFN-γ (interferon gamma), and chemokines which cause damage to the neurons and lead to their degeneration.

neurodegenerative disorders (160). It has been manifested that Aβ protein aggregation may trigger the initiation of NLRP3 in microglial cells [160, 161], which may promote the production of activated IL-1β and caspase-1. Further, it has been evidenced that microglial cells in animal models and AD subjects may exhibit enhanced levels of IL-1β and caspase-1 [136, 161, 162]. It has been documented that amyloid plaques instigate microglial cells to scavenge the Aβ-oligomers, which in turn triggers NLRP3 activation with a subsequent release of proinflammatory markers such as (IL-1β and IL-18) and several other neurotoxic factors. This process of liberation augments the neurotoxic effects of Aβ and aggravates the pathological processes of AD [161, 163–165]. Microglial cells are the prime components in the system which play a critical role in the inflammatory responses, but the abnormal microglia-specific NLRP3 activation may trigger chronic neuroinflammation [166].

4. Role of Inflammation on Aβ Pathology in AD

Inflammation is believed to be the prime cause of AD, and the deposition of toxic proteins inside the brain trigger inflammatory responses which lead to the aggravation of the disease. Injured or damaged regions of the brain tissues initiate inflammation, which is provoked by the AD toxic lesions and subsequent neuronal damage. The inflammatory process triggers the implementation and functioning of several inflammatory markers in response to injury or by other toxic events which are involved in inflammation. Early Aβ oligomer deposition in the brain may promote the activation of glial cells, enhanced levels of cytokines, and activation of the complement system [167]. Following the accumulation of Aβ peptides, an immune reaction in the brain triggers the release and activity of inflammatory markers such as TNF-α, IL-1, IL-6, and the activation of specialized brain cells [168, 169] The inflammatory process takes place in the brain having dense and abridged pathologies of AD, though there are minor cases of inflammation in the brain regions with reduced AD pathologies [25]. Transgenic animals were observed in an experimental study that depicted their execution of inflammatory cytokines which triggered several pathological alterations such as neuronal damage, demyelination, and activation of astrocytes and microglial cells [30]. Aβ production and its clearance are the two main events in the progression of AD, in which the ineffective clearance of Aβ in the brain leads to the accumulation of the toxic peptides that are associated with AD, although, microglia could help in the eviction of Aβ at the beginning and play an indispensable

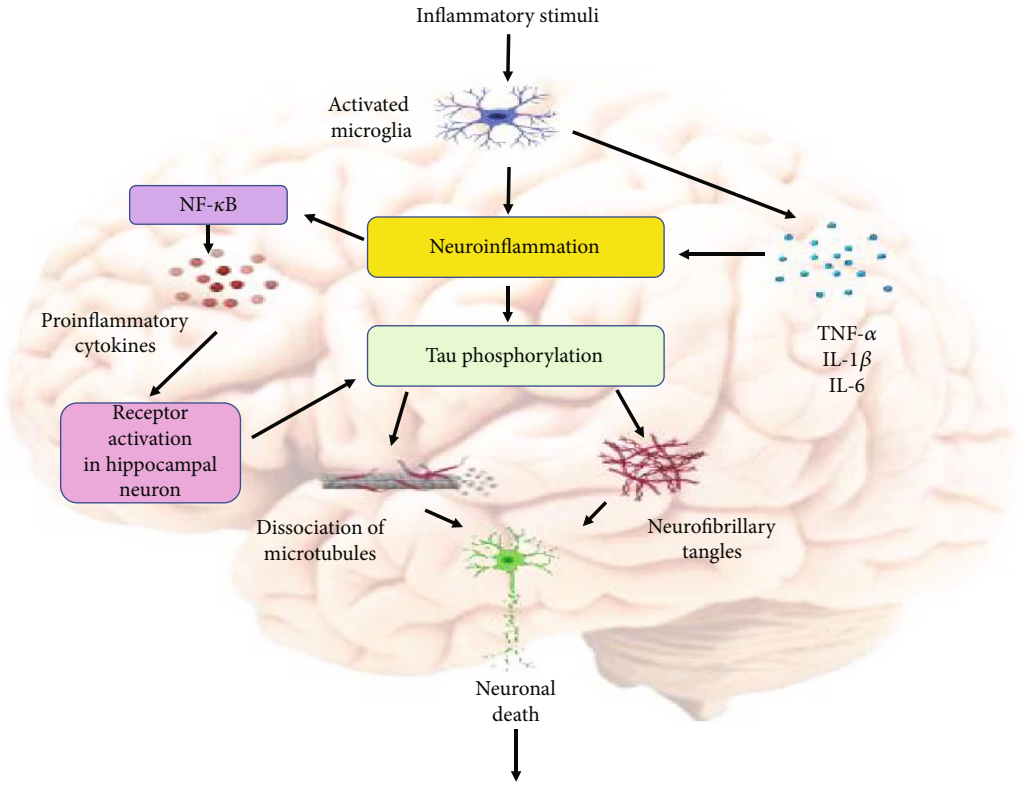

Figure 4: Role of inflammation on Tau pathology: inflammatory stimuli activate the microglial cells and trigger production of proinflammatory cytokines and Tau accumulation in the AD brain. Proinflammatory mediators such as TNF-α (tumor necrosis factor), IL-1β (interleukin-1β), and IL-6 (interleukin-6) could trigger neuroinflammation and tau pathology. Neuroinflammatory response activates a signaling cascade with the release and activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), overproduction of proinflammatory cytokines, and the activation of neuronal receptors. Hyperphosphorylation of tau protein initiates the dissociation of microtubule. Soluble tau aggregates into pathological tau oligomers, forms tau filaments, and ultimately leads to the formation of neurofibrillary tangles, which promote the neuronal death (created with http://BioRender.com/).

role in the inhibition of AD pathology. Hence, all these pieces of evidence support that inflammation is the key process in the AD pathology by which several proinflammatory cytokines enhance Aβ generation and the malfunction of specialized cells inside the brain, diminishing the levels of Aβ-degrading enzymes, and damaged BBB (Figure 5).

5. Prevention Is Better than Cure

Presently, no pharmacological therapy has been elucidated to be completely curable for AD. From the past few years, scientists, researchers, and the public have gained much interest in phytochemicals, as they proved to be effective in preventing various neurodegenerative diseases. For decades, traditionally acquired medicines from plants have been constantly implemented in the health care system in underdeveloped countries [3, 170]. Plant-derived medicines are in abundance with less toxicity and minimal health hazards and are more economical than synthetic drugs [171]. For various ailments, medicinal plants are being extensively used in traditional therapy since phytomedicines exhibit psychotropic, adaptogenic, and neuroprotective properties.

Phytochemicals possess anti-inflammatory, antioxidant, antiapoptotic, and neuroprotective properties and are broadly disseminated in the plant kingdom with minimum adverse effects [152, 172, 173]. Researchers have demonstrated that phytochemicals including nutraceuticals played a significant therapeutic role against AD. It has also been evidenced that the antioxidants vitamin C and E played a potential role against oxidative stress in AD patients, but more studies are needed to further know the detailed approaches of antioxidants [174, 175]. Several phytochemicals including curcumin, thymoquinone, lycopene, piperine, anthocyanins, and catechins used in both in vitro and in vivo animal models manifested anti-inflammatory and antioxidant properties [176–178]. In AD, neuroinflammation leads to a significant increase in the expression of inflammatory cytokines, including TNF-α, IL-6, and IFN-γ, and alterations in the levels of oxidative markers [41, 179, 180]. Researchers reported that supplementation of lycopene had no influences on peroxidation markers and cytokines in asthmatic and obese patients, but supplementation with polyphenols showed marked results in patients. However, green tea extract significantly ameliorated the inflammatory and oxidative markers in obese as well as in hemodialysis patients. Likewise, curcumin

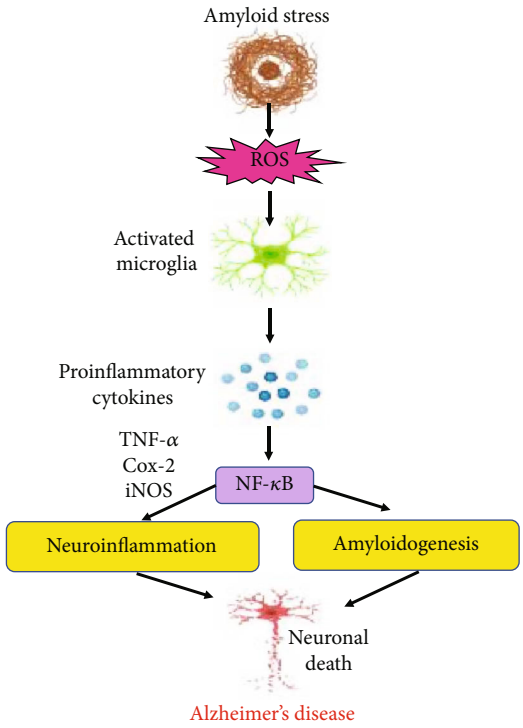

Figure 5: Role of inflammation on Aβ pathology: Aβ stress leads to the production of ROS, and the inflammatory stimuli activate the microglial cells, which leads to the production of proinflammatory cytokines and causes the elevated levels of cytokines and accumulation of activated microglia. The promoter region of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) has binding sites, which lead to the process of amyloidogensis and inflammation, and the activated NFκB initiates the production of proinflammatory cytokines, which triggers neuroinflammation. These mediators cause the excitation of the glial cells which further stimulate the production of Aβ burden and additional proinflammatory cytokines and ultimately leads to the death of neurons and AD pathology.

attenuated IL-1β and peroxidation markers in osteoarthritis subjects but showed no changes in obese patients.

The intervention of curcumin, grape juice, blueberry, coca flavanols, and green tea extracts ameliorated cognitive function in humans as well in mouse models [181, 182]. Polyphenolic nutraceuticals have been reported to provide neuroprotection through the modulation of misfolded amyloid-beta and hyperphosphorylated tau protein generation [183], initiation of inflammatory cytokines (TNF-α and IL-1β), modulation of MAPK and NF-κB pathways, and inception of nuclear factor-erythroid 2-related factor 2 (Nrf2) or antioxidant responsive elements. All these events lead to the activation of several enzyme cascades to deliver antitoxic and antioxidant properties [184]. Several phytochemicals from herbs, spices, and extracts of medicinal plants including terpenoids and triterpenoids trigger the modulation of NF-κB or initiation of Nrf2 nuclear factor [185, 186]. Several studies reported that antioxidant beverages and apple and green tea extracts have significantly attenuated the TNF-α and IFN-γ proinflammatory cytokine expression in AD patients at the initial stage, whereas expression levels of these cytokines were significantly upregulated at the moderate stage of AD [187]. However, IL-1α is in an increased level in the moderate stage of AD, but no significant changes were observed in the initial stage of AD, although antioxidant beverages also did not cause any significant improvement or alteration in the levels of IL-1α in both of the stages of AD [187]. Therefore, it is demonstrated that nutraceutical-provoked interactions between IκB, Iκ kinases, and electrophiles lead to the modulation of NF-κB and Nrf2 factors [188–190].

It is evidenced from several studies that flavonoid metabolites may play an essential role and serve as a precursor of drugs, thereby attenuating the generation of ROS and secretion of inflammatory cytokines [136, 156–194]. Although, naringenin flavonoids do not always trigger antioxidant and anti-inflammatory responses, naringenin-4-O-glucuronide, a predominant metabolite of naringenin, upregulated the expression of TNF-α and Nrf2 whereas other metabolites of naringenin-7-O-glucuronide attenuated the expression of Nrf2 factor [136, 158–195]. The blood-brain barrier (BBB) is the semipermeable membrane that inhibits nonselective crossing of solute into the CNS extracellular fluid and separates the brain from the periphery. It has been speculated that antioxidant and oxidative stress markers may not be detected inside the brain [196]. However, it has been observed in experimental studies that flavonoids do cross the BBB upon the oral induction of flavonoids to rodents, thereby allowing interaction; they were transported inside the brain by specific transporters expressed in the BBB [182, 197]. Various studies manifested that the ATP-binding cassette (ABC) induces multidrug resistance (MDR), and MDR transporters may contribute to AD pathology; the accumulation of Aβ fibrils in the brain [198] targeting these ABC transporters are being considered as the biomarkers for the amelioration of AD pathology [199]. Hence, it has been observed that acute consumption and induction of flavonoids may play a vital role in the modulation of MDR, via the activation protein-1 (AP-1), Nrf2, and NF-κB pathways [200]. Thus, flavonoids are considered an efficient therapeutic option and may play an essential role in the prevention of AD.

A study reported that the extract of turmeric combined with green tea and black pepper extract showed promising results against AD [201]. It has also been reported that piperine exhibited antioxidant and anti-inflammatory properties and offered neuroprotection, thus acting as a preventive agent against AD. Although its bioavailability is low because of internal metabolism and having hydrophobicity, the employment of a nanoparticle-mediated delivery system augments its bioavailability to be accessible as a suitable vehicle for the prevention of AD [202]. Curcumin is another herbal supplement that is effective against AD, thereby attenuating the inflammation-associated markers in AD. Further, it was revealed that curcumin plays a promising role in diminishing the Aβ-provoked neuroinflammation via PPARγ activation in AD rat models [203]. Curcumin has also been found to be actively associated with astrocyte and microglial regulation, and modulated the NF-κB pathway, as well as reduced the production of proinflammatory cytokines [204]. Having less bioavailability, it is also being encouraged to reach the

Table 1: Phytochemicals that affect neuroinflammation in animal and cellular AD models.

Name of the plant	Phytochemicals	Experimental model used	Mechanism of action	References
Turmeric, *Curcuma longa*	Curcumin	SQ-injected D-galactose-induced mouse model	It activates ERK/PKC-arbitrated CREB regulation and Akt/GSk3β-arbitrated regulation. Stimulates BDNF and regulates the levels of caspase-3, TNF-α, and NFκB	[206]
Tea plant, *Camelia sinensis*	Epigallocatechin-3-galate	Human astrocytoma U373MG cells	It regulates the activation of NFκB and MAPK; reduces the levels of IL-1, IL-6, IL-8, and Cox-2; promotes the secretion of BDNF and NGF; attenuates caspase-3 and ROS levels	[207]
Sweet orange, *Citrus sinensis*	Naringenin	Hypoxia rat model	It initiates the activation of Nrf2/ARE signaling; enhances the levels of antioxidants; attenuates the levels of NO, cytokines, and NFκB signaling	[208]
Mangosteen, *Garcinia mangostana*	α-Mangostin	C57BL/6J triple transgenic mouse model	Plays an essential role in the regulation of inflammatory process; enhances BDNF expression and attenuates the phosphorylation of tau; regulates the levels of IL-1β, TNF-α, and caspase-3	[209]
Indian pennywort, *Centella asiatica*	Asiatic acid	Aluminium-induced rat model	Asiatic acid attenuates the Aβ toxicity by reducing the levels of APP, Aβ1-42, and β- and γ-secretases. It also reduces the expression of inflammatory mediators in the hippocampus and cortex and enhances the expression of GFAP and Iba-1	[99]
Black seed, *Nigella sativa*	Thymoquinone	LPS/IFN-γ-activated BV-2 microglia	Inhibition of NFκB initiated neuroinflammation, suppression of inflammatory markers (NO, IL-1β, and TNF-α), and production by regulating PI3K/Akt/NF-κB signaling	[210]
Ginger, *Zingiber officinale*	Gingerol	ICV-STZ-induced mouse model	It ameliorates the cognitive and behavioral dysfunction and AD-like pathology. It enhances the α-secretase activity and attenuates cerebral Aβ-42, β-secretase, APH1a activity, and COX-2-associated neuroinflammation	[211]
Citrus, *Citrus* × *sinensis*	Hesperidin	Aβ-induced APPswe/PS1dE9 transgenic mouse model	It exhibits the inhibitory effect on inflammatory mediators (TNF-α, IL-1β, COX-2, and iNOS). It enhances antioxidant defense and improves cognitive function. It attenuates the Aβ pathology by reducing H_2O_2 levels and restoring depleting GSH levels and total antioxidant capacity	[212]
Grape vine, *Vitis vinifera*	Quercetin	SAMP8 (senescence model)	It protects neuronal cells by reducing oxidative stress and neuroinflammation. It inhibits Aβ aggregation and tau phosphorylation. It suppresses neuroinflammatory processes by decreasing proinflammatory cytokines (iNOS, COX-2, and IL-1β) and reduces the levels of GFAP in the hippocampus	[213]

desired site via executing several nanoparticles, thereby playing a significant role in the prevention of AD [205].

Curcumin has been reported to trigger the stimulation of BDNF and regulate the extent of TNF-α, NF-κB, and caspase-3. It has been shown to activate the ERK/PKC and Akt/GSk3β signaling pathway in a D-galactose-induced mouse model [206]. Epigallocatechin-3-galate has been found to regulate the initiation of NF-κB and MAPK pathways and reduce the levels of IL-1, IL-6, IL-8, and COX-2 inflammatory markers. It also exhibited the secretion of BDNF and NGF and reduced the levels of caspase-3 and ROS in human astrocytoma U373MG cells [207]. The naringenin compound was reported to promote the activation of Nrf2/ARE signaling, augment the levels of antioxidants, and reduce the extent of NO, cytokines, and NF-κB signaling in the hypoxia rat model [208]. The α-mangostin compound has been found to play an instrumental role in the regulation of inflammatory responses, augment the expression of BDNF protein, and reduce the phosphorylation of tau protein. It has also been shown to regulate the levels of inflammatory markers including IL-1β, TNF-α, and caspase-3 in the C57BL/6J triple transgenic mouse model [209]. It is manifested that Asiatic acid, a triterpenoid of *Centella asiatica*, diminished the expression of APP, Aβ1-42, β, and γ-secretases. Also, it has been found to attenuate the expression of inflammatory mediators in the hippocampus and cortex regions of the brain and enhance the expression of GFAP and Iba-1 proteins in the aluminium-induced AD rat model [99]. Thymoquinone, an extract of *Nigella sativa* was shown to inhibit the NF-κB triggered neuroinflammation and subsequently suppress the production of (NO, IL-1β, and TNF-α) inflammatory mediators by regulating the PI3K/Akt/NF-κB signaling pathway in LPS/IFN-γ activated BV-2 microglia [210]. Gingerol, a compound of *Zingiber officinale*, has been documented to ameliorate the cognitive, behavioral deficits, and AD-like pathology. It enhances the α-secretase activity and reduces cerebral Aβ-42-, β-secretase-, and COX-2-associated neuroinflammation in the ICV-STZ-induced mouse model [211]. Researchers revealed that hesperidin inhibits the stimulation of (TNF-α, IL-1β, COX-2, and iNOS) inflammatory mediators. It enhances the antioxidant defense, augments the cognitive function, and attenuates the Aβ pathology by reducing H_2O_2 levels and restoring reduced GSH levels and total antioxidant capacity in the Aβ-induced mouse model [212]. Quercetin is another phytochemical extracted from grape vine reported to provide protection to neuronal cells by diminishing oxidative stress and neuroinflammation. It has also been revealed that quercetin inhibits Aβ aggregation and tau hyperphosphorylation and regulates the neuroinflammatory process by reducing the levels of iNOS, COX-2, and IL-1β, and diminishes the levels of GFAP in the SAMP8 senescence model [213] (Table 1).

6. Conclusion and Future Prospective

Based upon the previous studies, it has been elucidated that inflammatory responses are the major cause and have been highly involved in the progression of AD. Biomarkers for the disease play an influential role in the earlier detection and intervention of pharmacotherapy for the effective prevention of inflammatory responses and the development of selective inhibitors that spot specific arbitrators in the inflammatory cascade. Astrocytes and microglial cells perform integral roles during the development and physiology of the brain. Astrocytes are involved in the maintenance of the CNS, which offers structural stability, receptive surface insulation, and cushioning of extracellular chambers. These supportive cells play a significant role against inflammation and proceed to divide and block impaired areas. Synaptic plasticity and synaptic transmission are being modulated by astroglial cells, thereby providing protection to neurons against toxicity, and metabolically deliver enough support to warrant their effective performance. In addition, astrocytes are also involved in the inception and the advancement of AD.

Several anti-inflammatory drugs have been administered against AD models, but to date, the anti-inflammatory drugs have not exhibited comprehensive protection against the disease. More studies are warranted to determine the anti-inflammatory responses and the use of the appropriate model to facilitate the positive effects of therapeutic drugs. In recent times, phytochemicals have intrigued and drawn enough attention for the development of new therapeutic drugs for the prevention of AD. The therapeutic approaches of polyphenolic compounds, nutraceuticals, and antioxidants have been well elucidated and have shown positive results against neuroinflammation in AD and various other neurodegenerative diseases. Researchers believe that phytochemicals and polyphenols may revive the AD pathologies as they have shown promising results against the disease and may represent as excellent preventive agents against AD by modulating pathways related to inflammation. Several polyphenols have been studied for therapeutic approach and efficiently exhibited positive results and are entertained as the best preventive agents against AD. However, more studies are required to develop phytochemicals as novel preventive vehicles for AD.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

MAR, AK, SA, HR, MUR surveyed the literature and wrote the original manuscript. MQ, SR, RMA, and MAK edited and organized the manuscript. Conceptualization was done by AK and MUR. MAR and AK contributed equally to this work. All authors have read and agreed to the published version of the manuscript. First authorship is shared by Mashoque Ahmad Rather and Andleeb Khan.

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Research Article

Saussureae Radix Attenuates Neuroinflammation in LPS-Stimulated Mouse BV2 Microglia via HO-1/Nrf-2 Induction and Inflammatory Pathway Inhibition

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The activation of microglial cells and their subsequent neuroinflammatory reactions are related to various degenerative brain diseases. Therefore, the regulation of microglial cell activation is an important point for the research of therapeutic agents for treating or preventing neurodegenerative disorders. Saussureae Radix (SR) is the root of *Saussurea lappa* Clarke, and it has been used for a long time as an herbal medicine in East Asia to treat indigestion and inflammation of the digestive system. In previous studies, however, the effect of SR ethanolic extract on microglial cell-mediated neuroinflammation was not fully explained. In this study, we explored the antineuroinflammatory activities and molecular mechanisms of SR in microglial cells stimulated with LPS (lipopolysaccharide). Our results illustrated that SR does not cause cytotoxicity and significantly weakens the production of nitric oxide (NO) and inflammatory cytokines. SR treatment also inhibited the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase- (COX-) 2, induced heme oxygenase- (HO-) 1, and activated the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway. In addition, SR significantly repressed the transcriptional activities of the nuclear factor- (NF-) κB and activator protein- (AP-) 1. Furthermore, SR effectively inhibited the phosphorylation of mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT). Isolation and high-performance liquid chromatography (HPLC) analysis indicated two major sesquiterpenoids (costunolide and dehydrocostuslactone). These compounds significantly inhibited the production of neuroinflammatory mediators and induced HO-1 expression. These findings show that SR could be a potential candidate for the treatment of inflammation-related degenerative brain diseases.

1. Introduction

Microglia are important immune cells, and they play critical roles in the central nervous system (CNS) in the brain. In normal conditions, they are continually scavenging the CNS for abnormal brain cells and pathogens, and they also help regulate synaptic homeostasis [1]. However, activated conditions of microglia give rise to neurotoxic substance production, such as inflammatory mediators and proteins, all of which are implicated in neurodegenerative disorders [2]. LPS acts as a prototypical endotoxin, inducing inflammation, sepsis, and death. LPS is thus commonly used to create *in vitro* models of inflammation [3]. In activated conditions upon LPS stimulation, MAPKs in turn mediate some signal pathways, such as the transcription factors NF-κB and AP-1 [4]. AP-1 and NF-κB are closely connected in the production of inflammatory molecules, including iNOS and cytokines [5]. In addition, the JAK/STAT is another important pathway with critical roles in immune responses via the release of growth factors and proinflammatory cytokines [6]. Accordingly, regulation of the MAPK, NF-κB, AP-1, and JAK/STAT pathways can be important for treating inflammatory diseases.

HO-1 is an enzyme that promotes the decomposition of heme into carbon monoxide (CO) and biliverdin. HO-1 is highly inducible, and it is expressed in many neuronal cells,

including HT22 and BV2 cells [7]. In addition, HO-1 inhibits the production of proinflammatory factors, such as NO and inflammatory cytokines [8]. HO-1 and its by-product CO decrease iNOS expression, thereby reducing the level of iNOS-derived NO [9]. HO-1 production is induced by activation of redox-dependent transcription factor Nrf-2 [10]. In the inflammatory process, free Nrf-2 translocates to the nucleus and consequently induces HO-1 production [11]. Thus, some recent studies identified the importance of HO-1 for immunomodulation or anti-inflammatory efficacy.

SR has long been used as a medicinal herb to treat various diseases of the digestive system. Previous studies reported that SR can ameliorate ethephon-induced reproductive toxicity in rats and alleviate house dust mite-induced atopic-like dermatitis in Nc/Nga mice [12, 13]. Also, another review study showed that SR exhibits anticancer, anti-inflammatory, antiulcer, and cholagogic effects [14]. However, the influence of SR and its molecular mechanisms on neuroinflammation in microglial cells remain unknown. Thus, the present study investigated the inhibitory effect of SR on neuroinflammation and its mechanisms using BV2 microglial cells upon LPS stimulation. To evaluate the antineuroinflammatory efficacy of major constituents in SR, we isolated two major sesquiterpenoids, costunolide (1) and dehydrocostuslactone (2). Furthermore, two isolated compounds were also investigated quantitatively using HPLC analysis, and we explored its antineuroinflammatory efficacy.

2. Materials and Methods

2.1. Materials and Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Hyclone (Logan, UT, USA). LPS, dexamethasone, bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 100 mm culture dishes and 6–96-well plates were obtained from Sarstedt (Nümbrecht, Germany). A cell-counting kit (CCK) was purchased from Dojindo (Kumamoto, Japan). Enzyme-linked immunosorbent assay (ELISA) antibody sets were obtained from eBioscience (San Diego, CA, USA). Various primary antibodies and horseradish peroxidase- (HRP-) conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA) and Novus Biologicals (Centennial, CO, USA). The Alexa Fluor 488-conjugated anti-rabbit secondary antibody was obtained from Invitrogen (Carlsbad, CA, USA). An RNA extraction kit was obtained from iNtRON Biotech (Daejeon, Korea). Oligonucleotide primers for real-time reverse transcription-polymerase chain reaction (RT-qPCR), DNA synthesizing kits, and AccuPower® 2x GreenStar qPCR Master Mix were obtained from Bioneer (Daejeon, Korea). SR ethanolic extract (used 100% ethanol) was obtained from the Korea Plant Extract Bank (Ochang, Korea).

2.2. Cell Culture, Test Drug Treatment, and Stimulation. BV2 cells were grown in DMEM (contains 1% antibiotics and 10% FBS) and incubated in a humidified 5% CO_2 atmosphere at 37°C. To stimulate the microglia, LPS (100 ng/mL) was used in the presence or absence of SR (1, 10, 50, or 100 μg/mL), costunolide (1, 10, or 25 μM), dehydrocostuslactone (1, 5, or 10 μM), or DMSO (0.1%, vehicle control (VC)).

2.3. Cell Viability Test. The potential cytotoxicity of SR was examined by CCK assay. BV2 cells were seeded and preincubated for 18 h, and various concentrations of SR, DMSO, or LPS were added to the cells. After incubation for 24 h, the CCK solution was added and incubated for an additional 1 h. Cell viability was calculated from the optical density at 450 nm using an ELISA reader.

2.4. Analysis of NO Secretion. NO levels were examined by measuring nitrite concentrations in the culture medium. BV2 cells were plated, preincubated with SR, costunolide, dehydrocostuslactone, or DMSO for 1 h, and stimulated with LPS for 24 h. Then, 100 μL of the Griess reagent was added to each well. After incubation at room temperature for 5 min, absorbance was measured at 570 nm using an ELISA reader.

2.5. Cytokine Determination. For ELISA, BV2 cells were seeded and incubated for 18 h. The cells were pretreated with several concentrations of SR, costunolide, dehydrocostuslactone, or DMSO for 1 h, then stimulated with LPS for an additional 6 h. The inflammatory cytokine levels of the culture medium were measured by the ELISA antibody set in accordance with the manufacturer's protocol.

2.6. Total RNA Extraction and RT-qPCR. Total RNA extraction and cDNA synthesis were carried out using easy-BLUE™ RNA extraction kits (iNtRON Biotech) and AccuPower® CycleScript RT PreMix (Bioneer), respectively, and the methods of previous studies were referred [15]. The experimental setting, specific methods, and PCR conditions for RT-qPCR referred to our previous research methods [15]. The sequence of oligonucleotide primers used in this study is shown in Table 1 [15]. The amplification and analysis were performed using a QuantStudio 6 Flex Real-time PCR System (Thermo Fisher Scientific, Rockford, IL, USA), and each sample was compared by the relative CT method. The results of RT-qPCR were presented as gene induction fold, which was calculated using the internal control.

2.7. Preparation of Whole-Cell, Cytosolic, and Nuclear Extracts. To obtain whole-cell lysates, pellets were resuspended in the radioimmunoprecipitation assay lysis buffer (Millipore, Bedford, MA, USA) containing protease and phosphatase inhibitors. Cytosolic and nuclear fractions were isolated using NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) as described by the manufacturer.

2.8. Western Blotting Analyses. Total proteins were normalized using Bradford's method [16]. The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking nonspecific binding sites using 3% BSA, membranes were incubated with each primary antibody at 4°C overnight. The membranes were subsequently incubated with each HRP-conjugated secondary antibody. Protein levels were

Table 1: Primers used for RT-qPCR.

Target gene	Primer sequence
TNF-α	F: 5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′
	R: 5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′
IL-6	F: 5′-TCCAGTTGCCTTCTTGGGAC-3′
	R: 5′-GTGTAATTAAGCCTCCGACTTG-3′
IL-1β	F: 5′-ATGGCAACTGTTCCTGAACTCAACT-3′
	R: 5′-CAGGACAGGTATAGATTCTTTCCTTT-3′
iNOS	F: 5′-GGCAGCCTGTGAGACCTTTG-3′
	R: 5′-GCATTGGAAGTGAAGCGTTTC-3′
COX-2	F: 5′-TGAGTACCGCAAACGCTTCTC-3′
	R: 5′-TGGACGAGGTTTTTCCACCAG-3′
HO-1	F: 5′-TGAAGGAGGCCACCAAGGAGG-3′
	R: 5′-AGAGGTCACCCAGGTAGCGGG-3′
β-Actin	F: 5′-AGAGGGAAATCGTGCGTGAC-3′
	R: 5′-CAATAGTGATGACCTGGCCGT-3′

F: forward; R: reverse.

quantified using a ChemiDoc™ Touch Imaging System (Bio-Rad). The information about the various primary and secondary antibodies is listed in Table 2.

2.9. Immunofluorescence Staining. BV2 cells were seeded and incubated overnight. After treatment with SR and LPS, BV2 cells were incubated for 1 (NF-κB p65) or 3 h (Nrf-2). Cells were washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature. After blocking, immobilized cells were incubated with each primary antibody overnight at 4°C, washed three times with PBS, and incubated with the Alexa Fluor 488-conjugated secondary antibody. Cells were incubated with 4,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature and observed under a fluorescence microscope [17].

2.10. Plant Material. Dried SR was kindly provided from Bomyeong Herbal Market, Seoul, in 2016. Its scientific name was identified by one of the authors (Dr. Wei Li). A voucher specimen (ID-160078) was deposited at the Herbarium of Korean Medicine (KM)-Application Center, Korea Institute of Oriental Medicine, Republic of Korea.

2.11. Extraction and Isolation. The dried SR (500.0 g) was reflux extracted three times using EtOH (1.5 L). The total extraction (80.0 g) of EtOH was suspended in deionized water and partitioned with EtOAc and water fraction, yielding EtOAc (1A, 38.0 g) and water (1B, 41.5 g). The EtOAc fraction was subjected to silica gel column chromatography with a gradient of hexane-EtOAc-MeOH (10 : 1 : 0, 9 : 1 : 0, 8 : 1 : 0, 6 : 1 : 0.1, 5 : 1 : 0.1, 4 : 1 : 0.1, 3 : 1 : 0.1, and 2 : 1 : 0.1, with 1.0 L for each step) to give 11 fractions (Fr. 1A-1-1A-11). Fraction 1A-2 was isolated with a gradient of H_2O-MeOH (1 : 4, 1 : 3, 1 : 2, and 1 : 1 and MeOH) by MPLC using the YMC C18 column to give 8 fractions (Fr. 1C-1-1C-8). The fraction 1C-2 was separated by a Sephadex LH-20 column and eluted by MeOH, and its subfraction was isolated by prep-HPLC to give compound 1 (460.0 mg; purity > 97%) and compound 2 (586.0 mg; purity > 95%).

2.12. Sample Preparation for HPLC Analysis. The standard stock solutions for HPLC were prepared by dissolving accurately weighed compounds in 100% methanol (1 mg/mL). SR extract was prepared at a concentration of 10 mg/mL. All solutions for analysis were filtered through 0.45 μm RC membrane syringe filters (Sartorius, Germany).

2.13. Optimization of Chromatographic Conditions. HPLC analysis was performed using a Dionex UltiMate 3000 system (Dionex Corp., Sunnyvale, CA, USA) equipped with a binary pump, autosampler, column oven, and diode array UV/VIS detector (DAD). System control and data analysis were carried out using Dionex Chromeleon software. Separation was carried out on a Luna C18 column (250 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA), with the column oven temperature kept at 30°C, at a UV wavelength of 200 nm. The mobile phase consisted of water (solvent A) and methanol (solvent B) with 25 : 75 at 0–20 min, 75%–100% of B at 20-30 min in a flow rate of 1.0 mL/min.

2.14. Validation of the Method. The linear calibration curves were plotted with diluted five different concentrations. Linearity was assessed by computing the correlation coefficient (R^2) of the calibration curve for two compounds. Five concentrations of compounds were analyzed in triplicate. Regression equations were calculated using the equation y

Table 2: Primary and secondary antibodies used for Western blotting analysis.

Antibody	Corporation	Product no.	RRID	Dilution rate
iNOS	Cell Signaling	#13120	AB_2687529	1 : 1000
COX-2	Cell Signaling	#4842	AB_2085144	1 : 1000
β-Actin	Santa Cruz	#SC-47778	AB_626632	1 : 1000
HO-1	Cell Signaling	#82206	AB_2799989	1 : 1000
Nrf-2	Novus Biologicals	#NBP1-32822	AB_10003994	1 : 1000
TBP	Cell Signaling	#8515	AB_10949159	1 : 1000
NF-κB p65	Cell Signaling	#8242	AB_10859369	1 : 1000
P-IκBα	Cell Signaling	#2859	AB_561111	1 : 1000
IκBα	Cell Signaling	#4814	AB_390781	1 : 1000
P-ERK	Cell Signaling	#4377	AB_331775	1 : 1000
ERK	Cell Signaling	#9102	AB_330744	1 : 1000
P-p38	Cell Signaling	#9211	AB_331641	1 : 1000
p38	Cell Signaling	#9212	AB_330713	1 : 1000
P-JNK	Cell Signaling	#9251	AB_331659	1 : 1000
JNK	Cell Signaling	#9252	AB_2250373	1 : 1000
P-c-Jun	Cell Signaling	#3270	AB_2129575	1 : 1000
c-Jun	Cell Signaling	#9165	AB_2130165	1 : 1000
c-Fos	Cell Signaling	#2250	AB_2247211	1 : 1000
P-JAK2	Cell Signaling	3771	AB_330403	1 : 1000
JAK2	Cell Signaling	3230	AB_2128522	1 : 1000
P-STAT1	Cell Signaling	9167	AB_561284	1 : 1000
STAT1	Cell Signaling	14994	AB_2737027	1 : 1000
P-STAT3	Cell Signaling	9145	AB_2491009	1 : 1000
STAT3	Cell Signaling	12640	AB_2629499	1 : 1000
2nd anti-mouse	Cell Signaling	#7076	AB_330924	1 : 5000
2nd anti-rabbit	Cell Signaling	#7074	AB_2099233	1 : 5000

$= ax \pm b$, where x and y are the concentration and peak area of the compound, respectively.

2.15. Statistical Analyses. All data are presented as the mean ± standard error of the mean of three independent experiments. Statistical significance was analyzed using one-way analysis of variance followed by Dunnett's test after comparing the LPS and each treated sample. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Influence of SR on the Viability of BV2 Cells. To examine the potential cytotoxic effects of SR on BV2 cells, CCK assays were conducted. As presented in Figure 1(a), treatment with SR (1–100 μg/mL) for 24 h caused no cytotoxicity, and slight proliferation was observed at concentrations of ≥50 μg/mL. Also, 0.1% DMSO used as a vehicle control had no significant effect on cell viability.

3.2. Effects of SR on the Secretion of NO and Cytokines. To investigate the antineuroinflammatory efficacy of SR, we first evaluated the NO production following LPS stimulation using Griess assay. As presented in Figure 1(b), LPS treatment strongly elevated NO levels, whereas pretreatment with SR strongly diminished NO production in a concentration-dependent manner. We next investigated the influences of SR on the LPS-induced inflammatory cytokine production and expression of their mRNAs using ELISA and RT-qPCR, respectively. As shown in Figures 1(c)–1(g), LPS-treated cells strongly increased the production of tumor necrosis factor- (TNF-) α and interleukin- (IL-) 6 as well as their mRNA levels, whereas SR treatment suppressed cytokine and mRNA levels in a concentration-dependent manner. In addition, treatment of 0.1% DMSO (vehicle control) did not affect the production of neuroinflammatory mediators.

3.3. Effects of SR on the Expression of iNOS and COX-2. We next researched the protein and mRNA expression of iNOS and COX-2, the synthesizing enzymes of NO and prostaglandin E_2, respectively. The results indicated that pretreatment with SR effectively suppressed LPS-induced iNOS and COX-2 expression at the protein and mRNA levels (Figures 2(a) and 2(b)).

3.4. Effects of SR on the Induction of HO-1 and Nuclear Translocation of Nrf-2. HO-1 was effectively induced by SR pretreatment even under LPS stimulation. HO-1 protein expression was strongly induced by ≥50 μg/mL SR, and its mRNA expression was induced by 100 μg/mL SR

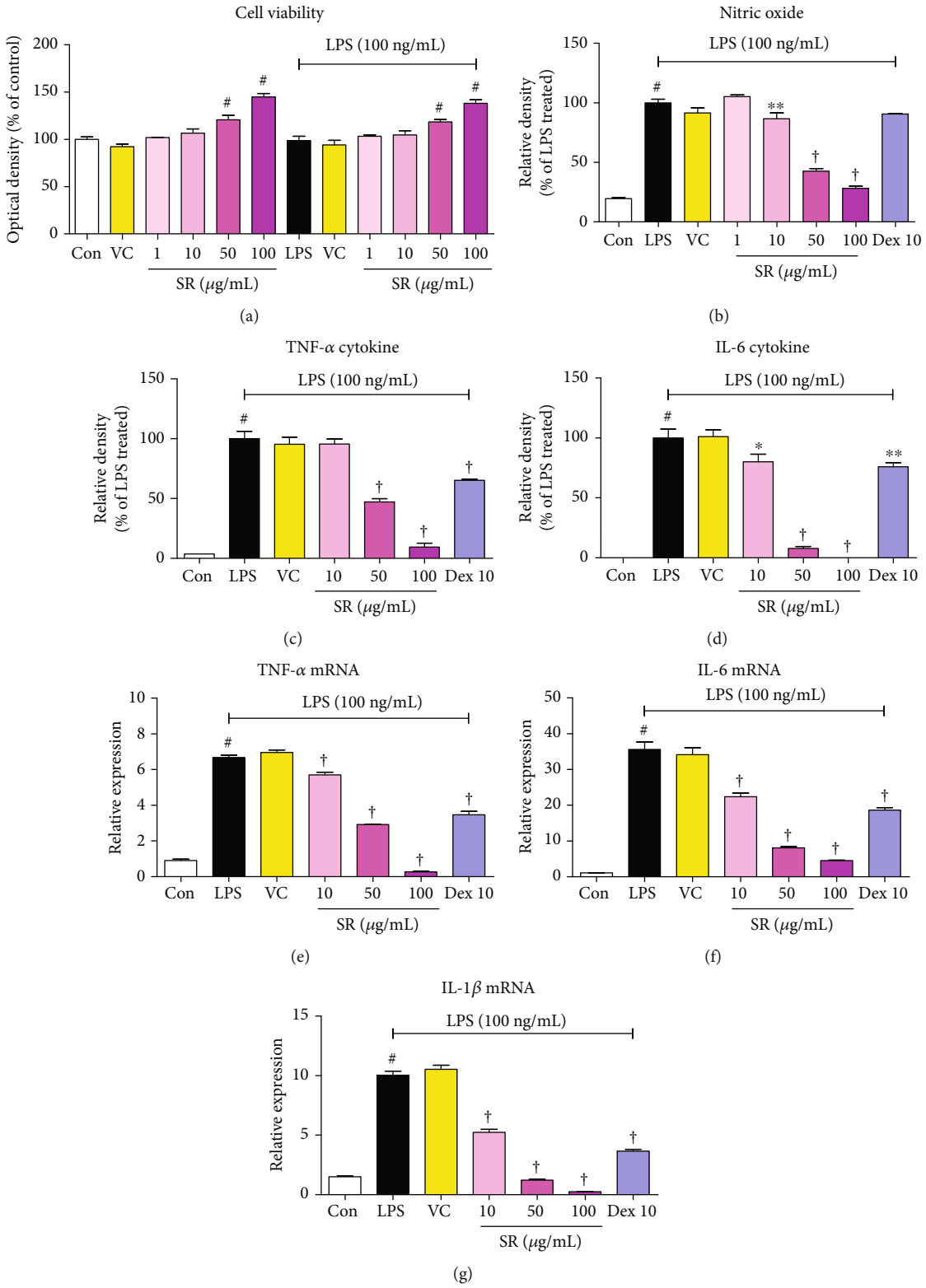


Figure 1: Effects of Saussureae Radix (SR) on (a) viability of microglia, secretion of (b) nitric oxide (NO) and (c, d) inflammatory cytokines, and (e–g) expression of cytokine mRNA in BV2 cells. Control cells were incubated with only fresh DMEM. BV2 cells were incubated with SR, vehicle control (VC; 0.1% dimethyl sulfoxide (DMSO)), or stimulated with lipopolysaccharide (LPS) for 24 (a–d) or 12 h (e–g). TNF: tumor necrosis factor; IL: interleukin. $^{\#}p < 0.05$ (vs. control); $^{*}p < 0.05$ and $^{\dagger}p < 0.001$ (vs. LPS).

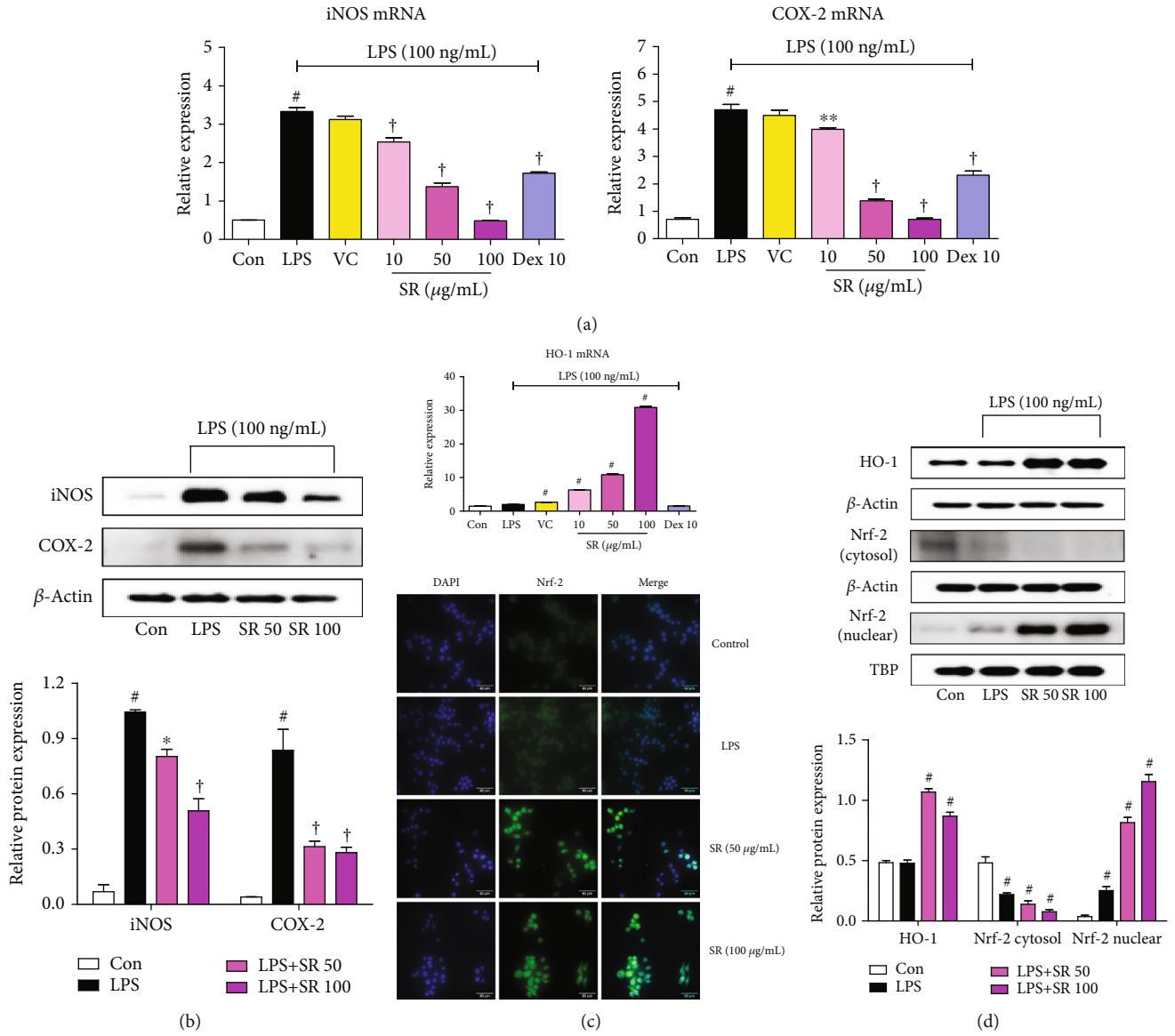


Figure 2: Effects of Saussureae Radix (SR) on (a, b) the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase- (COX-) 2, (c) the mRNA expression of heme oxygenase- (HO-) 1, and (d) the protein expression of HO-1 and nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf-2). Control cells were incubated with only fresh DMEM. BV2 cells were stimulated with lipopolysaccharide (LPS) for 12 (iNOS and COX-2), 6 (HO-1 protein), or 3 h (HO-1 mRNA and Nrf-2). (b, d) The histogram graphs show protein expression levels relative to those of the housekeeping protein. (d) The cultured BV2 microglia were incubated with anti-Nrf-2 (green) and DAPI (blue). Fluorescence was developed using the Alexa Fluor 488-conjugated anti-rabbit secondary antibody. $^{\#}p < 0.05$ (vs. control); $^{*}p < 0.05$ and $^{\dagger}p < 0.001$ (vs. LPS).

(Figures 2(c) and 2(d)). The nuclear translocation of Nrf-2, which is the molecular mechanism that regulates HO-1 induction, was also elevated by SR, and the extent of translocation was increased with increasing SR concentrations (Figure 2(d)). We also used immunofluorescence imaging to confirm the effect of SR on the nuclear translocation of Nrf-2 in BV2 cells and obtained results similar to those of Western blotting (Figure 2(d)).

3.5. Effects of SR on the Transcriptional Activity of NF-κB. Because the NF-κB pathway plays a pivotal role in the neuroinflammatory process, we examined the inhibitory efficacy of SR on the phosphorylation of IκBα and NF-κB transactivation in LPS-stimulated microglia. Western blotting revealed that SR effectively inhibited p65 translocation from the cytoplasm to the nucleus in a concentration-dependent manner (Figure 3(a)). Additionally, SR strongly decreased the phosphorylation of IκBα upon LPS stimulation (Figure 3(a)). Moreover, the nuclear translocation of p65 as identified using immunofluorescence imaging was strongly suppressed by SR pretreatment, in line with the Western blotting result (Figure 3(a)).

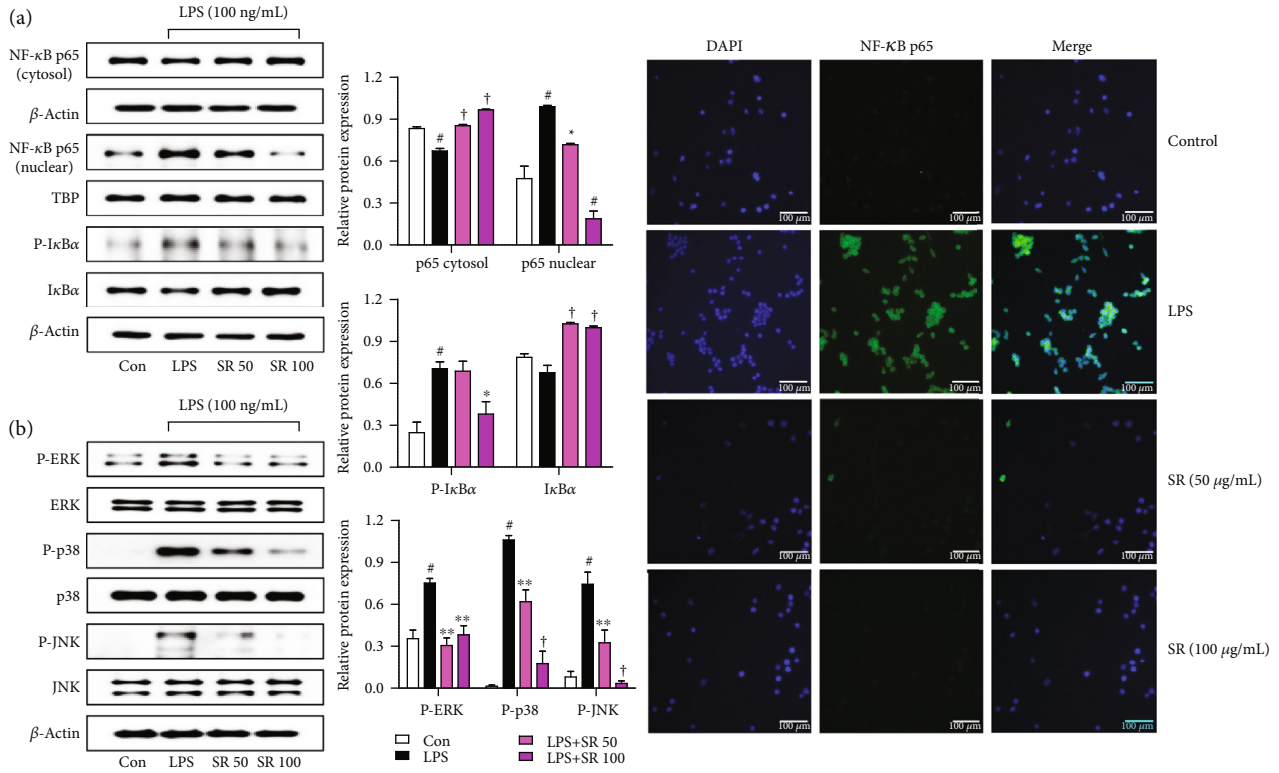


FIGURE 3: Effects of Saussureae Radix (SR) on the (a) nuclear translocation of nuclear factor- (NF-) κB p65 and phosphorylation of inhibitor of NF-κB alpha (IκBα) and (b) phosphorylation of three mitogen-activated protein kinases (MAPKs). Control cells were incubated with only fresh DMEM. Cells were stimulated with lipopolysaccharide (LPS) for 1 h (NF-κB p65) or 30 min (IκBα and MAPK). The histogram graphs show protein expression levels relative to those of the housekeeping protein. (a) The cultured BV2 microglia were incubated with anti-NF-κB p65 (green) and DAPI (blue). Fluorescence was developed using the Alexa Fluor 488-conjugated anti-rabbit secondary antibody. $^{\#}p < 0.05$ (vs. control); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\dagger}p < 0.001$ (vs. LPS).

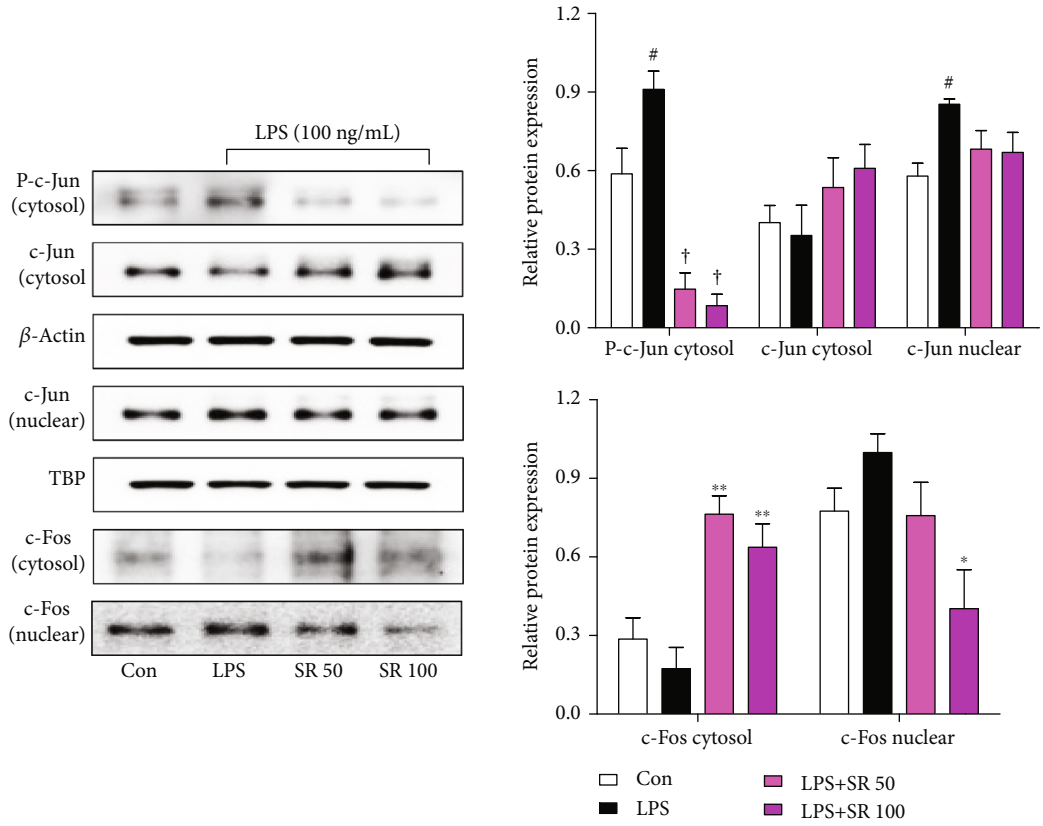
3.6. Effects of SR on MAPK Phosphorylation. The phosphorylation of MAPK is involved in the production of many proinflammatory factors. Therefore, we analyzed the change of the phosphorylation of three MAPKs including extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK). Results of Figure 3(b) indicated that SR pretreatment repressed the activation of MAPKs, including ERK, p38, and JNK.

3.7. Effects of SR on LPS-Induced AP-1 Pathway Activation. AP-1 migrates to the nucleus and regulates the expression of certain inflammatory genes in response to inflammation caused by stimuli, such as LPS [6]. We therefore measured the level of AP-1 phosphorylation in the cytoplasm and nuclear AP-1 levels. As presented in Figure 4(a), LPS stimulation induced c-Jun phosphorylation and its migration to the nucleus, whereas pretreatment with SR inhibited the nuclear transfer and phosphorylation of c-Jun. The nuclear translocation of c-Fos, another subunit of AP-1, was also suppressed in a similar manner by SR pretreatment (Figure 4(a)).

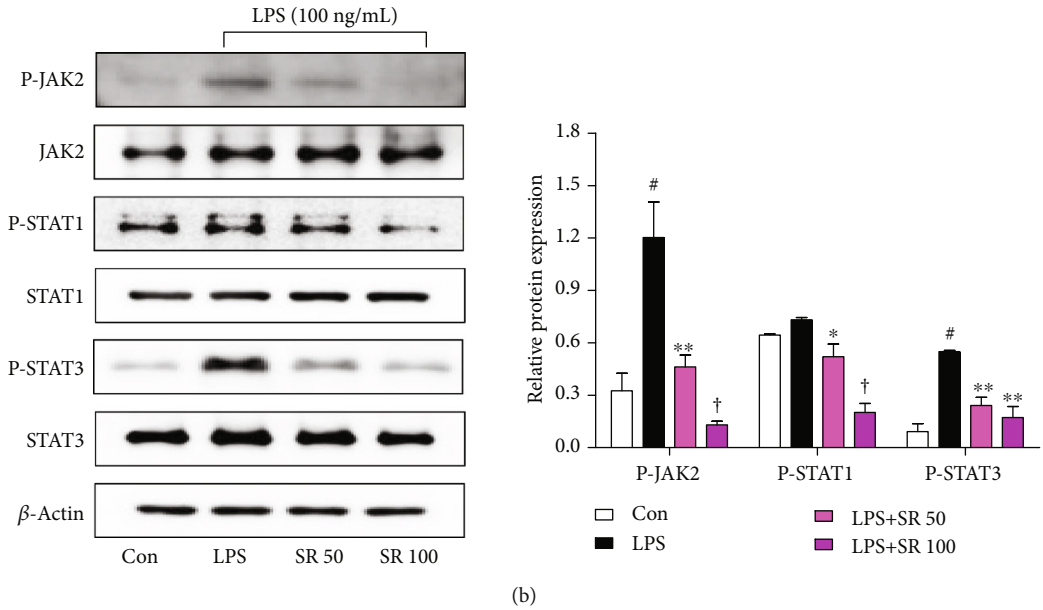
3.8. Effects of SR on Activation of the JAK/STAT Pathway. Previous research reported that mitigation of the JAK/STAT pathway inhibited the secretion of NO and proinflammatory cytokines [18]. Therefore, we measured the inhibitory effects of SR on the phosphorylation of JAK2, STAT1, and STAT3 in LPS-stimulated BV2 cells. As presented in Figure 4(b), SR treatment significantly blocked the phosphorylation of JAK2, STAT1, and STAT3 without affecting their total protein levels.

3.9. Isolation and Structural Elucidation. Using combined chromatographic separation techniques, costunolide (1) and dehydrocostuslactone (2) were effectively isolated from SR (Figure 5). The HPLC evaluation showed that the purity of the isolated compounds was >95%. The structures were confirmed by comparing the obtained spectroscopic data with the previously reported values [19].

3.10. Verification of Antineuroinflammatory Effects of Costunolide and Dehydrocostuslactone in BV2 Microglia upon LPS Stimulation. To investigate the cytotoxicity of the main constituents of SR, we carried out CCK assays on microglia. As shown in Figure 6(a), costunolide showed no cytotoxicity at concentrations below 25 μM and dehydrocostuslactone at concentrations below 10 μM. The two components showed slight cytotoxicity at concentrations greater than 25 μM and 10 μM, respectively, so subsequent experiments used only concentrations below that. Also, both costunolide and dehydrocostuslactone effectively suppressed the production of NO and cytokines induced by LPS stimulation



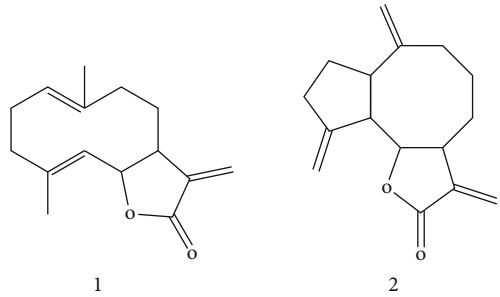
(a)



(b)

Figure 4: Effects of Saussureae Radix (SR) on the (a) nuclear translocation and phosphorylation of c-Jun and c-Fos and (b) phosphorylation of Janus kinase (JAK2), signal transducer and activator of transcription 1 (STAT1), and STAT3. Control cells were incubated with only fresh DMEM. Cells were stimulated with lipopolysaccharide (LPS) for (a) 1 or (b) 4 h. The histograms show protein levels relative to those of the internal control. $^{\#}p < 0.05$ (vs. control); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\dagger}p < 0.001$ (vs. LPS).

(Figures 6(b)–6(d)), and each mRNA expression was significantly and concentration-dependently suppressed (Figures 6(e)–6(g)). In addition, as shown in Figures 6(h)–6(j), the mRNA levels of iNOS and COX-2 were strongly suppressed by the pretreatment of costunolide and dehydrocostuslactone, and the expression of the antioxidant enzyme HO-1 mRNA was effectively induced. The 0.1% DMSO used as a vehicle control had no effect on cell viability and the production of neuroinflammatory mediators. In most of the above results, costunolide and dehydrocostuslactone showed



1 2

Figure 5: Structure of costunolide (1) and dehydrocostuslactone (2) isolated from SR.

concentration-dependent effects with statistical significance, respectively.

3.11. Identification of the Components of SR Using HPLC-DAD Analysis. The mobile phase consisted of methanol and water. Two standards and SR were detected using a PDA detector (200-400 nm). A wavelength of 200 nm was selected according to their maximum wavelength. By comparison of retention times (tR) and UV spectra of two standards (costunolide (1) (9.39 min) and dehydrocostuslactone (2) (10.68 min)) and SR, two compounds were detected (Figure 7).

3.12. Validation of the Analytical HPLC Method. The calibration curves for two compounds were obtained via plotting the peak area versus the concentration. The linear correlation coefficient (R^2) for the calibration curves was greater than 0.999 (Table 3). According to the calibration curves, the amounts of two compounds were found to be 62.81996 ± 0.0186 and 19.63455 ± 0.0042 mg/g, respectively.

4. Discussion

Microglial cell-mediated neuroinflammation has been identified as an important risk factor for neurodegenerative diseases. Overactivation of microglial cells leads to neuronal damage, brain injury, and the release of various neuroinflammatory mediators [20]. Reducing levels of these endogenous inflammatory factors through regulation of microglial activation is important in preventing and treating neuroinflammation [21]. SR is the dried root of *Saussurea lappa* Clarke (Compositae) and is native to the Himalayas in India. SR is listed in the ancient medicine text "Shennong's Classic of Materia Medica" and has long been used in East Asia to treat digestive disorders such as indigestion, vomiting, stomachache, diarrhea, and chronic inflammation of the digestive system. SR has also been used in the treatment of dysentery and testicular inflammation, and some recent studies have shown that SR can ameliorate reproductive toxicity in rats and alleviate house dust mite-induced atopic dermatitis in mice [12, 13]. In addition, a recent review study described that SR exhibits anticancer, anti-inflammatory, antiulcer, and cholagogic effects [14]. Furthermore, another recent study shows that SR has antioxidant and antineuroinflammatory efficacy [22]. However, the above work used ethyl acetate fraction of SR and demonstrated inhibitory activity for NO, iNOS, and TNF-α cytokine production along with 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity. Our present study demonstrated the inhibitory activity of SR on the generation of neuroinflammatory factors such as IL-6, IL-1β, and COX-2 as well as NO in BV2 cells activated by LPS stimulation. We also revealed the molecular mechanisms of anti-neuroinflammatory activity through investigating the effects of SR on the NF-κB, MAPK, AP-1, and JAK/STAT pathways. Additionally, we have identified the efficacy of SR for activation of the HO-1/Nrf-2 antioxidant pathway.

First of all, we conducted a CCK assay to exclude the potential cytotoxicity of SR to BV2 microglial cells, and SR did not affect cell viability up to 100 μg/mL. Furthermore, the proliferation of BV2 cells was confirmed when more than 50 μg/mL of SR was treated (Figure 1(a)). NO is a free radical that has been involved in the microglial cell-mediated inflammatory process in the CNS [23]. NO is synthesized from L-arginine by iNOS, and overproduction of NO is related to the presence of some inflammatory diseases and autoimmune disorders [24, 25]. NO secretion and iNOS expression levels were closely related to HO-1 induction [26], and HO-1 is regulated by Nrf-2. We therefore examined whether SR pretreatment inhibited the production of NO, inflammatory cytokines, iNOS, and COX-2 during the neuroinflammatory process upon LPS stimulation in microglial cells. Results of these experiments demonstrated that SR significantly reduced the production of NO, inflammatory cytokines, and its mRNAs without cytotoxicity (Figure 1) and inhibited the expression of iNOS and COX-2 protein and mRNA with statistical significance (Figures 2(a) and 2(b)). We further examined the effect of SR treatment on the induction of HO-1 and activation of Nrf-2. We found that SR markedly induced HO-1 expression and gradually increased the nuclear translocation of Nrf-2 with statistical significance (Figures 2(c) and 2(d)). The efficacy of SR for HO-1 induction and Nrf-2 activation appears to have an indirect effect on regulating the neuroinflammatory response by inhibiting NO and iNOS.

The activation of NF-κB and MAPK is associated with the pathogenesis in various diseases of CNS. NF-κB is the main regulator of various genes involved in immune and inflammatory reactions [27]. Depending on the activation of microglia by LPS, IκBα is degraded and phosphorylated, in which free NF-κB is moved to the nucleus and activates inflammatory mediators [28]. MAPK plays an important role in the expression induced by LPS of some endogenous inflammatory mediators and the activation of NF-κB [29, 30]. We examined whether the influence of SR on inflammatory mediators is associated with the change of NF-κB. Our results showed that treatment of SR efficiently repressed the nuclear translocation of NF-κB p65 in a concentration-dependent manner (Figure 3(a)). In addition, treatment of SR inhibited the phosphorylation and degradation of IκBα in a similar pattern (Figure 3(a)). We also investigated the effects of SR on the phosphorylation of MAPK proteins following LPS stimulation, revealing that MAPK activation was effectively suppressed by SR treatment through inhibiting phosphorylation of ERK, p38, and JNK (Figure 3(b)).

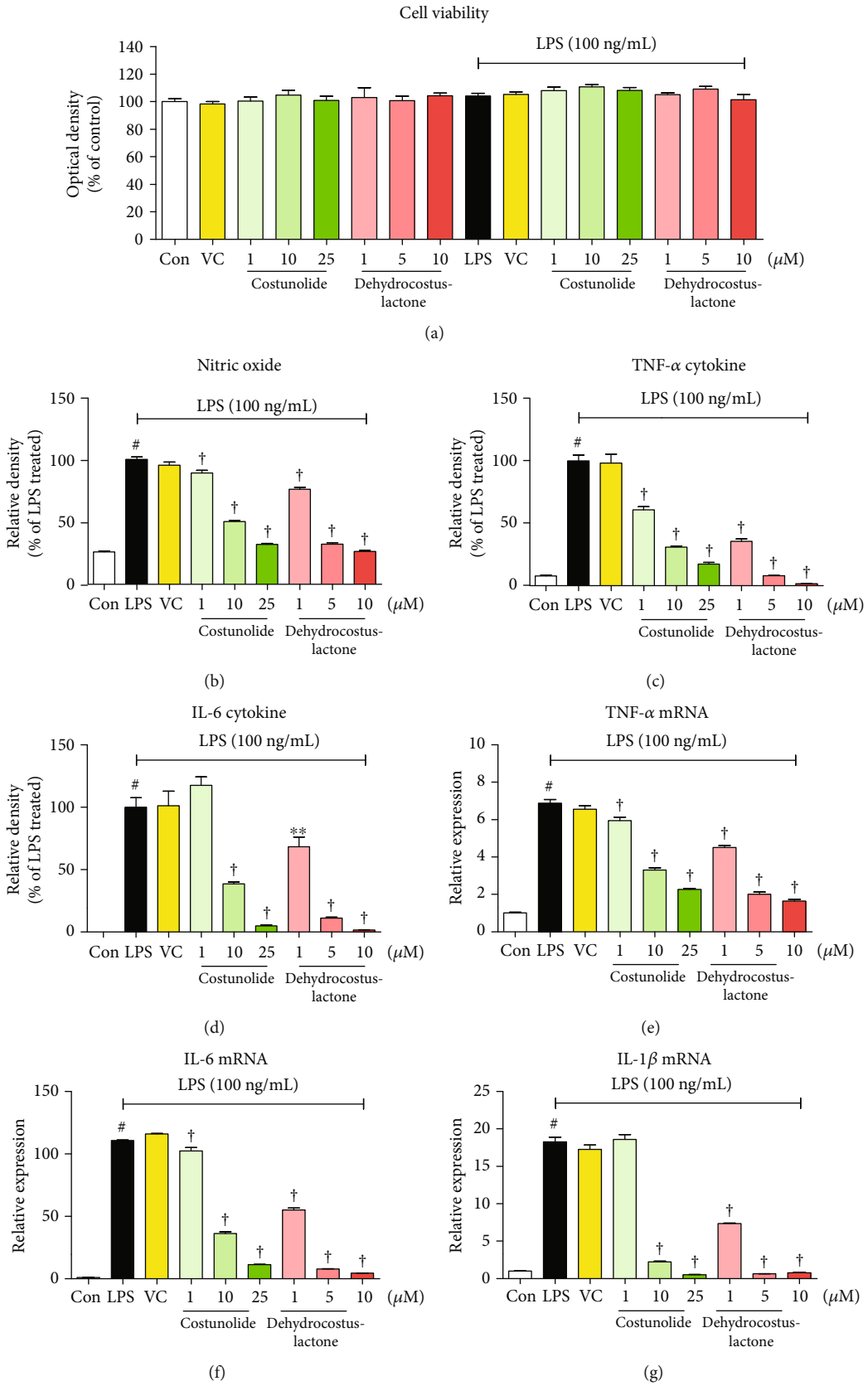


Figure 6: Continued.

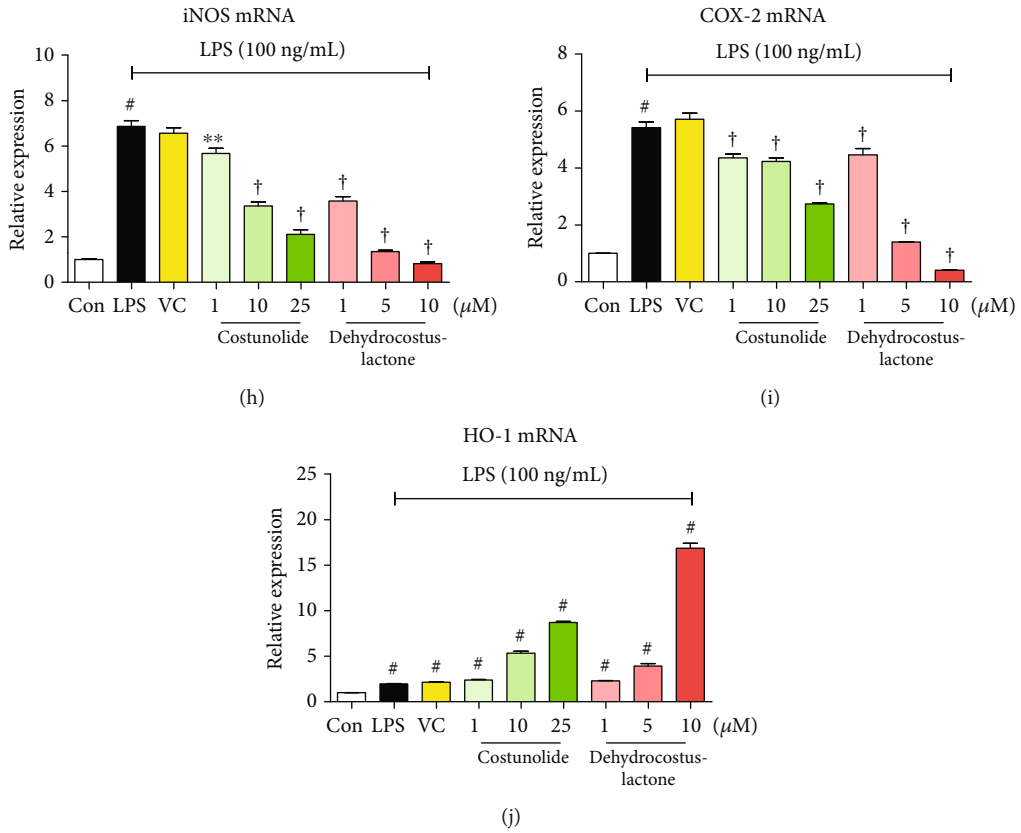
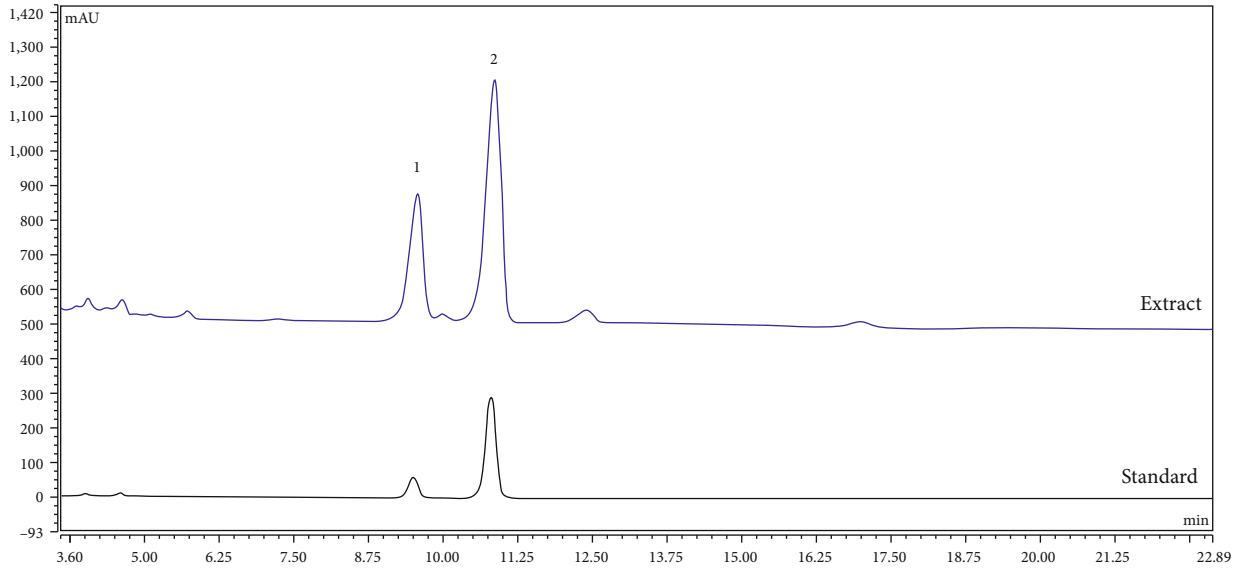


Figure 6: Effects of two compounds costunolide and dehydrocostuslactone on (a) viability of microglia, secretion of (b) nitric oxide (NO) and (c, d) inflammatory cytokines, and expression of (e–g) cytokine mRNAs, (h, i) inducible nitric oxide synthase (iNOS) and cyclooxygenase- (COX-) 2 mRNAs, and (j) heme oxygenase- (HO-) 1 mRNA. Control cells were incubated with only fresh DMEM. BV2 cells were incubated with each compound, 0.1% dimethyl sulfoxide (DMSO), or stimulated with lipopolysaccharide (LPS) for 24 (a, b), 6 (c–g), 3 (h, i), or 6 h (j). $^{\#}p < 0.05$ (vs. control); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\dagger}p < 0.001$ (vs. LPS).

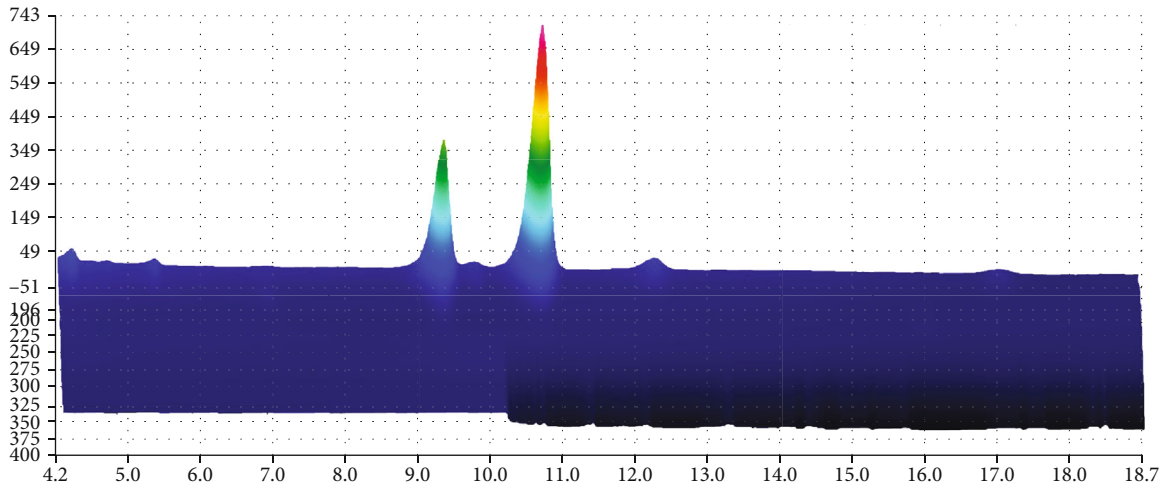
These results indicated that the inhibitory effects of SR on neuroinflammation might be controlled via the blockade of the activation of NF-κB and MAPK pathways.

The activation and nuclear translocation of AP-1 subunits regulate the expression of some inflammatory genes in inflammatory responses induced by LPS [6]. The JAK/-STAT pathway is known to elicit the production of various inflammatory mediators through phosphorylation and is recognized to have a central role in inflammatory responses [6, 31]. In addition, the phosphorylation of STAT3 has a direct effect on IL-6 secretion [32]. We therefore examined the effects of SR on the phosphorylation of c-Jun and nuclear transfer of c-Jun and c-Fos following LPS treatment, and the results demonstrated that SR pretreatment inhibited both phosphorylation and nuclear translocation of c-Jun and suppressed translocation into the nucleus of c-Fos (Figure 4(a)). In addition, SR pretreatment effectively inhibited the phosphorylation of JAK2, STAT1, and STAT3 (Figure 4(b)). These results indicated that the antineuroinflammatory activity of SR was attributable to both the blockade of the NF-κB and MAPK pathways and the inhibition of the AP-1 and JAK/STAT pathways.

We also identified the two main components (costunolide and dehydrocostuslactone) of the SR using HPLC analysis. Previous studies of these components have shown that costunolide ameliorates acute lung injury via attenuating MAPK and improved acute ulcerative colitis in mice through inactivation of NF-κB, STAT1/3, and Akt [33, 34]. There have also been reports that dehydrocostuslactone suppresses LPS-induced macrophage activation through NF-κB, p38 MAPK, and Akt [35]. Also, dehydrocostuslactone has been researched to be effective in inhibiting LPS-induced inflammation *in vitro* and improves the survival of mice in cecal ligation and puncture-induced sepsis *in vivo* [36]. These studies indicated that the antineuroinflammatory effect of SR is closely connected with the efficacy of its constituents, costunolide and dehydrocostuslactone. To confirm the antineuroinflammatory activity of these compounds, we investigated the effect of costunolide and dehydrocostuslactone on the production of NO, proinflammatory cytokines, inflammatory mRNAs, and antioxidant enzyme HO-1 on the microglia. Our results indicated that these compounds inhibited the production of NO and inflammatory cytokines including TNF-α and IL-6 without cytotoxicity at each concentration (Figures 6(a)–6(d)). Also, two compounds suppressed the expression of cytokine, iNOS, and COX-2 mRNA in a concentration-dependent manner (Figures 6(e)–6(i)). In addition, costunolide and dehydrocostuslactone strongly induced HO-1 mRNA expression (Figure 6(j)). Based on the above results, the regulatory



(a)



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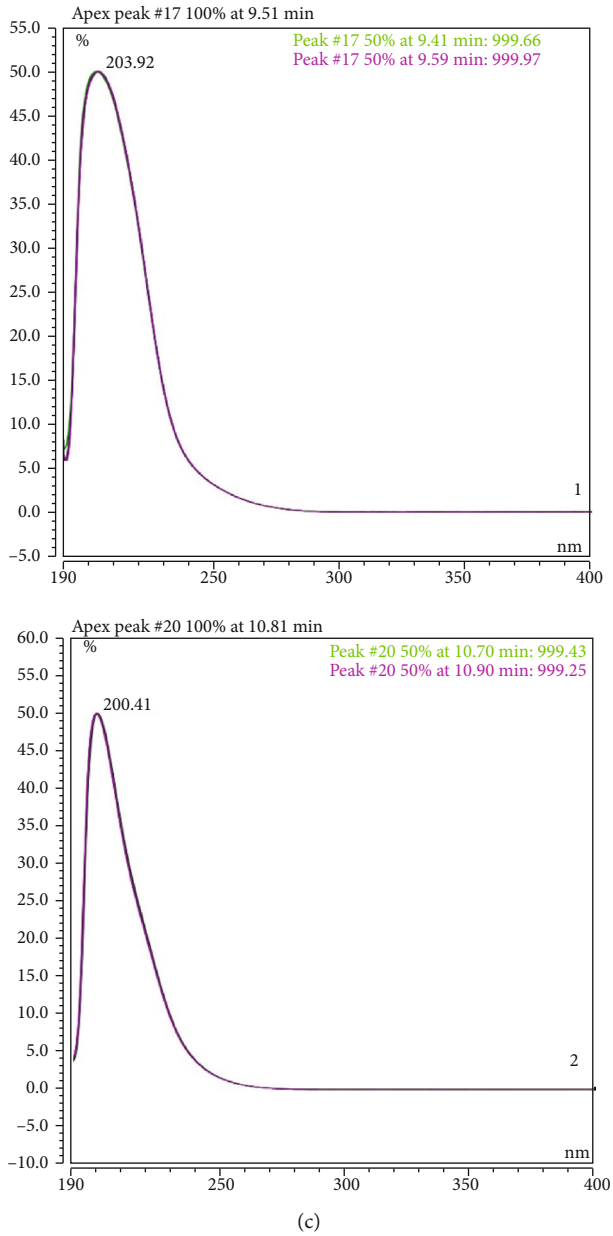

(c)

Figure 7: HPLC chromatograms ((a) 2D; (b) 3D) of two standard compounds from the ethanolic extract of SR at UV wavelengths of 200 nm (c). Costunolide (1) (9.39 min) and dehydrocostuslactone (2) (10.68 min) were identified.

Table 3: Regression data and contents of two compounds in SR.

Analytes	Regression equation	R^2	Content (mg/g)
Costunolide (1)	$y = 0.173x + 0.0257$	0.9999	62.81996 ± 0.0186
Dehydrocostuslactone (2)	$y = 1.0619x + 9.5492$	0.9994	19.63455 ± 0.0042

efficacy of SR on the neuroinflammatory response is thought to be closely related to the inhibitory efficacy of costunolide and dehydrocostuslactone on the production of inflammatory mediators.

The results of this study showed that SR contains anti-neuroinflammatory properties *in vitro* by attenuating inhibiting inflammatory mediators in LPS-stimulated BV2 cells. These beneficial activities are associated with enhanced HO-1/Nrf-2 activation and inhibition of the NF-κB/MAP-K/AP-1/JAK/STAT signaling pathways. The antineuroinflammatory effects of SR appear to be closely related to the presence of two components, costunolide and dehydrocostuslactone. Based on these results, SR appears to have potential value as a candidate for the treatment of inflammation-related degenerative brain diseases.

Abbreviations

AP: Activator protein
BSA: Bovine serum albumin
CCK: Cell-counting kit
CNS: Central nervous system
CO: Carbon monoxide
COX: Cyclooxygenase
DAD: Diode array UV/VIS detector
DAPI: 4,6-Diamidino-2-phenylindole
DMEM: Dulbecco's modified Eagle's medium
DMSO: Dimethyl sulfoxide
ELISA: Enzyme-linked immunosorbent assay
ERK: Extracellular signal-regulated kinase
FBS: Fetal bovine serum
HO: Heme oxygenase
HPLC: High-performance liquid chromatography
HRP: Horseradish peroxidase
IL: Interleukin
iNOS: Inducible nitric oxide synthase
JAK: Janus kinase
JNK: c-Jun NH_2-terminal kinase
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
NF: Nuclear factor
NO: Nitric oxide
Nrf-2: Nuclear factor erythroid 2-related factor 2
PBS: Phosphate-buffered saline
RT-qPCR: Real-time reverse transcription-polymerase chain reaction
SR: Saussureae Radix
STAT: Signal transducer and activator of transcription
TNF: Tumor necrosis factor
VC: Vehicle control.

Data Availability

The data used to support the findings are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

You-Chang Oh developed the study design, performed the experiments, analyzed the data, wrote the draft manuscript, and critically revised the manuscript. Wei Li performed the experiments, analyzed the data, and performed the phytochemical analysis. Jang-Gi Choi performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

Acknowledgments

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