ISSN: 2538-5887; Pathobiology Research. 2025; 28 (1): 7-28

Microglia in Parkinson's Disease: A Dual Role of M1 and M2 Phenotypes in Neuroinflammation

ARTICLE INFO

Article Type:
Original Paper

Authors:
Ehsan Baghban[1]
Masoud Soleimani[2*]

1. Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
2. Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran Medical Nanotechnology and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

**** Corresponding author:***
Masoud Soleimani

E-mail: soleim_m@modares.ac.ir

ABSTRACT

As the global population continues to age, the prevalence of Parkinson's Disease (PD) is increasing. PD is the second most common neurodegenerative disorder, particularly among the elderly. Its key symptoms include tremors, shaking, movement difficulties, and challenges with balance and coordination. The disease is characterized by the loss of dopaminergic neurons in a specific part of the brain, the substantia nigra pars compacta, and the aggregation of the α-synuclein protein within cells. In recent years, research has highlighted the significant role of inflammatory processes in PD pathology. However, it remains unknown If neuroinflammation is a cause or consequence of PD. Strong evidence suggests that microglia, the resident immune cells in the central nervous system, play a crucial role in protecting neurons and that dysfunctional and overly activated microglia are present in the brains of individuals with PD. Under normal conditions, microglia are in a "homeostatic" state, but in response to disease-related triggers, they transition to a "reactive state." The transition of microglial phenotypes can result in either pro-inflammatory (M1) or anti-inflammatory (M2) states, each characterized by distinct markers and released substances. Prolonged activation of the M1 phenotype is associated with a range of inflammatory conditions, including neurodegenerative diseases such as Parkinson's disease. Consequently, further investigation into the role of microglia is essential for enhancing our understanding of and therapeutic approaches to PD. This review will delve into the involvement of microglia in the neurodegenerative process of PD and explore the impact of microglia-mediated inflammation on the disease.

Keywords:

Microglia, Neuroinflammation, Parkinson's disease, Neurodegeneration.

INTRODUCTION

The first identification of Parkinson's disease (PD) occurred in 1817, when James Parkinson described the symptoms, which included tremor, rigidity, and postural instability, in his elderly patients in his work, 'An Essay on the Shaking Palsy' (1). The most prevalent movement disorder in the elderly is Parkinson's disease, with a prevalence of 0.5 to 1% in adults aged 60–69 and 1–3% in individuals over 80 years old. It is believed that more than 10 million individuals worldwide have PD, and this number is projected to grow to 30 million by 2030 (2, 3). Parkinson's disease is a commonly diagnosed degenerative neurological condition characterized by motor symptoms such as stiffness, slowness of movement, shaking at rest, and difficulty with balance, as well as non-motor symptoms such as reduced sense of smell, problems with the autonomic nervous system, and sleep disturbances. Pathologically, PD is marked by the gradual loss of dopamine-producing neurons in the substantia nigra pars compacta (SNpc) (4).

Along with the SNpc, there is a neural loss in the cerebral cortex, basal ganglia, thalamus, locus coeruleus, and brain stem, particularly the dorsal motor nucleus. The cellular aggregates found in dopaminergic neurons, known as Lewy bodies, and their primary protein component, α-synuclein (α-Syn), are the prominent neuropathological features of this disease (5). Research has shown that various molecular pathways can influence the progression of PD. Dysfunction in proteins produced by PD-associated genes can lead to neuropathology similar to sporadic PD through mechanisms such as genes, α-syn protein

regulation, neurotransmitters, autophagy, mitochondrial dysfunction, and oxidative stress. While age plays a crucial role in the development of PD, the cause of the disease is still not fully understood (6). However, more than 10% of PD cases are associated with familial forms of the condition, which have been linked to mutations in specific PD-related genes such as SNCA (PARK1), LRRK2 (PARK8), PRKN (PARK2), PINK1 (PARK6), and DJ-1 (PARK7). These gene mutations lead to autosomal recessive and autosomal dominant forms of familial PD. Extensive genome-wide association studies (GWAS) have demonstrated the involvement of certain genes associated with Parkinson's disease in the sporadic form of the condition. However, the factors that contribute to the onset of idiopathic Parkinson's disease, which accounts for approximately 90% of cases, remain inadequately understood. This situation emphasizes the critical need for additional research to elucidate the underlying mechanisms involved in disease development (7, 8). Idiopathic PD shows increased expression of normal α-syn despite the fact that the most common mutations in familial PD are not found in the SNCA gene. One common pathological feature observed in PD patients is the formation of inclusions due to the abnormal accumulation of α-syn protein inside cells (9). Currently, two types of PD models are utilized in research: neurotoxin-induced and transgenic models. Neurotoxin-induced models employ chemicals like rotenone, 6-hydroxydopamine (6-OHDA),1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and paraquat to induce symptoms similar to PD. Transgenic models involve the genetic modification of PD-related genes such as SNCA, LRRK2, PINK1, PRKN, and DJ-1 (10). In recent years, there has been an increase in interest in neuroinflammation as a mechanism involved in PD pathology. With age, the brain's homeostasis is disrupted because the activation of survival mechanisms decreases. Consequently, neuroinflammation may develop in PD. Neuroinflammation is a crucial immune response in the central nervous system (CNS) to preserve neuronal health. However, its neurotoxicity could worsen neuronal injury. Nonetheless, it is unclear whether neuroinflammation is a cause or a consequence of PD. Therefore, further research is necessary to elucidate the role of neuroinflammation in the mechanisms underlying PD pathology (11, 12). Researchers first used silver carbonate staining to identify microglia over a hundred years ago. Specific to the central nervous system, these cells serve an immune function akin to macrophages and engage with neurons while carrying out various tasks in the CNS. Accumulated findings indicate that microglia closely interact with neurons and impact the generation of neurotrophic factors like IGF-1, the process of neurogenesis, and the formation of synapses during brain development. Consequently, the crucial role of microglia in immune processes and brain balance is highlighted. Nevertheless, it is known that microglia's overactive and unregulated behavior can lead to harmful effects on the nervous system. Recent research has revealed that the activation of microglia and the molecules associated with it are linked to the advancement of neurodegenerative conditions such as epilepsy, Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease (HD) (13, 14). In this review, we will focus on the role of microglia in the neurodegenerative process of PD and discuss the contribution of microglia-mediated inflammation to PD with scientific studies.

Innate immune system and inflammaging

The extension of the human lifespan is considered a remarkable achievement of our time, yet it has brought about new challenges in ensuring the well-being of older individuals. Aging is the primary risk factor for various chronic age-related conditions, including PD. While the exact cause of aging remains unclear, there is growing recognition of low-level chronic inflammation, known as inflammaging, as a prominent feature associated with aging (15). It is still uncertain whether inflammatory pathways directly drive the aging process and contribute to PD progression or if they serve reparative and regenerative functions. Throughout evolution, the innate immune system has played a crucial role in maintaining internal stability (16). As the most evolutionarily conserved defensive mechanism, this system enables the organism to promptly identify and defend against threats by sensing and responding to infection or internal stress indicators through pattern recognition receptors (PRRs) encoded in the germ line. The activation of PRRs by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular

patterns (DAMPs) initiates downstream signaling pathways, leading to the production of cytokines and the activation of adaptive immunity (17). Parkinson's disease is a chronic neurodegenerative condition commonly found in elderly individuals. Although it has not traditionally been associated with inflammation, recent findings have linked PD to DAMPs-related sterile inflammation and neuroinflammation. This neuroinflammation is now recognized as a characteristic feature of PD and a common factor in its progression. Despite the long-held belief that the central nervous system is immune-privileged, our understanding of the influence of the immune system on brain function and the brain's regulation of peripheral immune functions remains limited. There is increasing recognition of the critical role played by immune cells responsible for maintaining tissue balance, including brain-resident macrophages, microglia, and circulating myeloid cells, in both brains aging and the progression of PD (18, 19).

Inflammation in PD: Cause or Consequence of the Disease

The central nervous system (CNS) has historically been regarded as an immune-privileged tissue due to several factors: (a) the absence of dendritic cells, (b) the presence of an immunosuppressive microenvironment within the brain parenchyma under normal physiological conditions, and (c) the existence of the blood-brain barrier (BBB), which separates the brain parenchyma from the peripheral immune system (20). However, the CNS can elicit an immune response when confronted with threats like pathogens or internal danger signals. This response is initiated by microglia, the resident macrophages of the CNS, which various stimuli can activate. All inflammatory responses must be resolved to maintain tissue structure and homeostasis, which involves the removal of pathogens, dead cells, and other cellular debris, along with restoring tissue integrity. Chronic inflammation may ensue if a threat persists or the mechanisms responsible for resolving inflammation are inadequate. Inflammation can also be triggered by molecules released from degenerating neurons, a process known as neuroinflammation, which plays a significant role in neurodegenerative diseases (21).

Growing evidence suggests that impairment of the blood-brain barrier (BBB) is a prevalent characteristic of neurodegeneration associated with neuroinflammation. BBB dysfunction has been documented in individuals with various neurodegenerative disorders, including Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and multiple sclerosis (22). The BBB is a complex structure that regulates the passage of specific plasma proteins and immune cells from the bloodstream into brain tissue. It is believed that the breakdown of the BBB facilitates the entry of lymphocytes, macrophages, and plasma proteins into the brain, which can trigger microglial activation and contribute to neurodegeneration. Various neuroimaging agents and techniques for detecting BBB dysfunction and neuroinflammation in neurodegenerative diseases have been extensively reviewed in the literature (19, 23). For instance, Ju et al. demonstrated that mannitol-induced BBB permeability enhancement leads to microglial activation. These findings indicate that a compromised BBB plays a critical role in microglial activation, enhancing our understanding of how to mitigate the exacerbation of sterile brain inflammation initiated by innate immune responses in glial cells. When glial cells are activated without disrupting the functionality of the brain endothelial barrier, this activation can result in BBB impairment due to the release of inflammatory mediators. Consequently, the compromised BBB permits the entry of blood-derived factors that further stimulate glial activation within the brain, escalating brain inflammation and perpetuating additional glial activation. Therefore, BBB dysfunction not only contributes to the progression of neuroinflammation but also serves as a crucial factor in this process rather than merely a pathological consequence of glial activation and neuroinflammation (24). Whether neuroinflammation acts as a cause or a consequence of these diseases remains uncertain.

Origin and function of microglia

Microglia functions as the brain's immune cells, serving as its defense system against threats to neural tissue. Over a century ago, Pío Del Río Hortega identified microglia using a silver carbonate staining technique to locate them in brain tissue samples. Hortega referred to microglia as "the true third element," alongside neurons and astrocytes. He described microglia as

a heterogeneous cell type that displays a variety of morphologies, ranging from highly ramified to ameboid forms, indicating the presence of different sub-populations of microglia (25, 26). Grasping the origins of various brain cells is crucial, as it lays a foundational understanding of their unique functions within the nervous system. Neurons, astrocytes, and oligodendrocytes, which originate from the ectoderm, and microglia, which originate from the mesoderm, each have distinct roles (27). Erythromyeloid progenitors (EMPs) in the yolk sac serve as precursor cells for microglia, setting them apart from hematopoietic stem cell (HSC)-derived macrophages. During early development, EMPs generated migrate throughout the body. Those that reach the liver, epidermis, lungs, and CNS differentiate into Kupffer cells, Langerhans cells, alveolar macrophages, and microglia (28). In most tissues, HSC-derived monocytes are eventually replaced by differentiated macrophages. However, due to the blood-brain barrier formed during development, HSC-derived macrophages are not replaced in the brain. Studies using Flt3 marker-labeled fetal and adult HSCs indicate that only 2% of brain microglia in 1-year-old mice are derived from HSCs. Identified by specific internal proteins such as Iba1, CD11b, and C-X3-C motif chemokine receptor 1 (CX3CR1), microglia are widespread in the CNS (28, 29). The variations in marker expression based on subtype and activation state are crucial for neuroscience studies. Microglia, similar to macrophages, serve as immune cells in the central nervous system. They clear foreign substances and dead cells, release chemokines and cytokines, and rapidly eliminate unnecessary substances, thereby supporting healing in affected areas and maintaining CNS homeostasis. However, their immune function can also lead to damage in progressive neurodegenerative diseases and chronic inflammatory responses (30, 31). Activated microglia have been recognized as significant contributors to the progression of various neurodegenerative diseases. In specific circumstances, microglia exhibit surprising changes in shape and behavior based on their external environment. Under normal conditions, microglia are in a resting state, characterized by elongated protrusions extending from their small cell body. Following nerve injuries, inflammation, or ischemia, they become activated, leading to the enlargement of the cell body and the shrinking of the protrusions, resulting in an appearance similar to macrophages (32). Microglia serve various functions, including extending their projections into neuronal synapses, monitoring the state of neurons through direct contact, and participating in synaptic pruning during central nervous system development. They can either promote or inhibit the progression of various CNS diseases. Moreover, the activation status of microglia within the same disease can be diverse and complex based on the extent of disease progression and the affected brain region. Previous pathological observations suggest the existence of different subtypes of microglia, each performing distinct functions (33, 34).

Microglia phenotypes

Under normal physiological conditions, microglia are usually found in a state of equilibrium, with a branched appearance, forming extensive connections with other cells such as neurons, oligodendrocytes, astrocytes, and blood vessels. They continuously monitor their surroundings for changes and can detect signs of damage in their immediate environment. When the nervous system is compromised, it transitions to an activated state in response to pathological triggers. The activities of microglia are often described based on pro-inflammatory (M1) and anti-inflammatory (M2) characteristics. Upon activation, microglia migrate to the locus of injury or infection and assume a dual role in the immune response, which can either facilitate healing or aggravate the condition. The regulation of the transition of microglia from a state of equilibrium to an activated state involves a complex interplay of factors (35, 36).

Although commonly categorized as M1 and M2, it is believed that multiple types of activation states and various targets and receptors are involved in regulating the response of microglia. The functional states of microglia form a spectrum rather than distinct phenotypes, and these characteristics are usually temporary rather than permanent. When activated, microglia typically exhibit an amoeboid shape, shorter processes, an enlarged cell body, and the expression of new cell surface receptors. They also release pro-inflammatory and anti-inflammatory factors that can either exacerbate or slow down the progression of diseases. In healthy

conditions, immune responses are finely regulated to maintain tissue equilibrium during initiation or resolution. In contrast, immune responses are uncontrolled during pathological conditions and prone to excessive imbalance, which can lead to cell loss or dysfunction. The communication of cytokines between the blood and the brain, influenced by infection and immune system substances produced by the endothelial cells of the blood-brain barrier, as well as peripheral immune signals released by the autonomic nervous system, continuously affects microglia. Additionally, the proper functioning of the central nervous system's immune system depends entirely on the complex, yet not fully understood, interactions between the peripheral and central nervous systems (35, 37, 38).

M1 phenotype

The M1 phenotype indicates the state of classical activation. When referring to microglia in this state, we use the term "M1 microglia." This state represents the body's initial line of defense and is characterized by microglia's pro-inflammatory and killing-promoting properties. Microglia secrete pro-inflammatory cytokines (such as IL-1β, IL-6, IL-12, IL-17, IL-18, IL-23, TNFα, and IFNγ), NO, and chemokines such as CC-chemokine ligand 2 (CCL2) when polarized to the M1 phenotype. Furthermore, microglia exhibit phenotypic markers after M1 activation, including major histocompatibility complex class II (MHC II), inducible nitric oxide synthase (iNOS), CD86, cyclooxygenase-2 (COX2), and additional molecules such as ROS, prostaglandin E2 (PGE2), and reactive nitrogen species (RNS). The coordinated activities are postulated to eliminate foreign pathogens and other threats while stimulating T cells to trigger an adaptive immune response (39, 40). In neurodegeneration, the neurotoxic M1 phenotype of microglia suggests a possible transition from the protective M2 phenotype to the harmful M1 phenotype or a replacement of the M1 phenotype by the M2 phenotype during the disease (41). Accumulation of α-synuclein aggregates, amyloid beta plaques, and other unidentified stimuli may trigger microglial M1 phenotypic expression. Microglia activated by these triggers are initiated by the MAPK-associated protein-1 (MAPK/AP-1) and inhibitor of kappa B/NF-κB (IκB/NF-κB) signaling cascade and produce pro-inflammatory cytokines (41, 42). Lipopolysaccharide (LPS) is widely recognized as an inflammatory stimulus for microglia. Studies have demonstrated that LPS induces M1 microglia through toll-like receptors (TLRs), leading to the demise of dopaminergic neurons in vivo and in vitro. The activation of LPS occurs following its binding to toll-like receptor 4 (TLR4) and toll-like receptor 2 (TLR2) on the microglial membrane, subsequently attaching to myeloid differentiation protein 2 (MD2) (TLR4/MD2), with the participation of the co-receptors CD14 and LPS binding protein (LBP). LPS activates TLR4, which then sets off a series of downstream signaling molecules, such as MyD88 and TRIF, along with transcription factors like NF-κB, STAT5, and IRFs. This sequence of events leads to an elevated transcription of M1-associated genes, including chemokines, cytokines, and genes that contribute to pro-inflammatory responses (43- 45). IFNγ, known as type II interferon, is crucial in innate and adaptive immunity against viral, bacterial, and protozoal infections. Activation of macrophages/microglia and induction of MHC II molecule production are important functions of IFNγ. It induces the M1 phenotype of microglia through the JAK/STAT signaling pathway by binding to IFNγ receptors 1 and 2 (IFNγR1/2), which then phosphorylate JAK1/2. Phosphorylated JAK1/2 activates STAT1, leading to its translocation to the nucleus and IRFs. This signaling pathway triggers the transcription of genes encoding cytokines, chemokines, and other molecules associated with the M1 inflammatory response (46-48). Additionally, GM-CSF has been found to stimulate M1 activation alternatively via the CD11b receptor. Unlike LPS and IFNγ, GM-CSF can induce various activation states, exhibiting characteristics of both the M1 and M2 phenotypes (49).

M2 phenotype

The term "M2 microglia" encompasses two distinct states: "alternative activation" and "acquired deactivation." These specialized microglia play a crucial role in a wide range of processes, including regulating the immune system, dampening inflammatory responses, promoting healing, and facilitating recovery from injury. Upon exposure to anti-inflammatory stimuli such as IL-4, IL-10, IL-13, TGFβ, and glucocorticoids, microglia transition to the M2 phenotype, which is closely linked to the

reduction of inflammation and the restoration of equilibrium. Following classical activation, a prompt anti-inflammatory and reparative phase is initiated to mitigate inflammation, repair tissue damage, and regenerate the extracellular matrix. Additionally, the "acquired deactivation" of the M2 phenotype serves as another mechanism to reduce acute inflammation, typically induced by engulfing apoptotic cells or exposure to TGF-β and IL-10 (anti-inflammatory cytokines) (50, 51). The morphological disparities and coexistence of the two phenotypes remain currently undisclosed. However, it is conceivable that in diverse environments, the amalgamation of these two phenotypes could contribute to the manifestation of inflammatory conditions associated with neurodegenerative disorders (52). M2-polarized microglia secrete anti-inflammatory substances such as arginase-1 (Arg1), which support tissue healing and extracellular matrix formation. These cells also produce growth factors such as FIZZ1, IGF-1, and chitinase 3-like 3 (Ym1), which aid in extracellular matrix deposition. Furthermore, M2-polarized microglia are characterized by M2 phenotype receptors on their cell surfaces, including triggering receptors expressed on myeloid cells, two receptors (TREM2), and the mannose receptor (CD206). Microglial activation significantly influences the pathophysiology of neuroinflammatory and neurodegenerative diseases, including Alzheimer's, Parkinson's disease, as well as other conditions such as amyotrophic lateral sclerosis, multiple sclerosis, traumatic brain injury, and stroke (53-55). The concept of microglia polarization remains the subject of ongoing debate as the M1/M2 paradigm may oversimplify in vivo activation. The heterogeneity of microglial phenotypes in various pathogenic contexts can be further elucidated through transcriptomic and proteomic analyses. M2-polarized microglia can be classified into three subtypes: M2a, M2b, and M2c, each characterized by distinct markers and triggers (56). IL-4 or IL-13 can activate the M2a state, which is associated with phagocytosis and tissue recovery. IL-4 binding to different receptors induces the transcription of specific genes related to the M2a state, such as CD206, suppressor of cytokine signaling 3 (SOCS3), and scavenger receptors (SRs). This state represents the primary pathway for microglial activation (57, 58). The M2b state is activated when both TLRs and IL-1 receptors are involved. It gives rise to the production of both pro- and anti-inflammatory cytokines (TNFα, IL-6, and IL-10) and assists in recruiting regulatory T cells to the inflammation site. Activation of M2b leads to the release of IL-10 and the expression of CD86 and MHC II on the cell surface (59, 60). The M2c state is implicated in the body's anti-inflammatory and healing processes. It is triggered by IL-10 and glucocorticoid hormones, suppressing pro-inflammatory cytokines associated with the M1 phenotype. Additionally, it is involved in tissue remodeling and matrix deposition (61). The shift from M1 to M2 phenotype, which involves transitioning from a pro- Figure 1. M1/M2 polarization state of microglia and their functions. Homeostatic microglia can be activated towards the proinflammatory (M1) and anti-inflammatory (M2) phenotypes. M1 microglia release proinflammatory cytokines, while M2 microglia produces anti-inflammatory cytokines. Moreover, M1 can be converted into M2 microglia by various inducers. M1 or M2 microglia can release different substances, and the microglia of the two phenotypes can be converted to each other under certain conditions.

inflammatory state to a regulatory/anti-inflammatory state, significantly improves functional outcomes and restores bodily equilibrium. When microglia are exposed to certain substances such as IL-10, glatiramer acetate, beta interferons, and others, they can transition from the M1 to the M2 phenotype. Although M1 and M2 microglial phenotypes have distinct functions, both M1- and M2-related factors can coexist simultaneously (50, 62, 63).

Furthermore, a mixed M1/M2 phenotype expression may occur in an injury environment due to different subpopulations expressing different phenotypes. Neurodegenerative conditions typically involve neuroinflammation and promoting the shift from M1 to M2 through pharmacological means could be crucial for treatment in these cases (Figure 1).

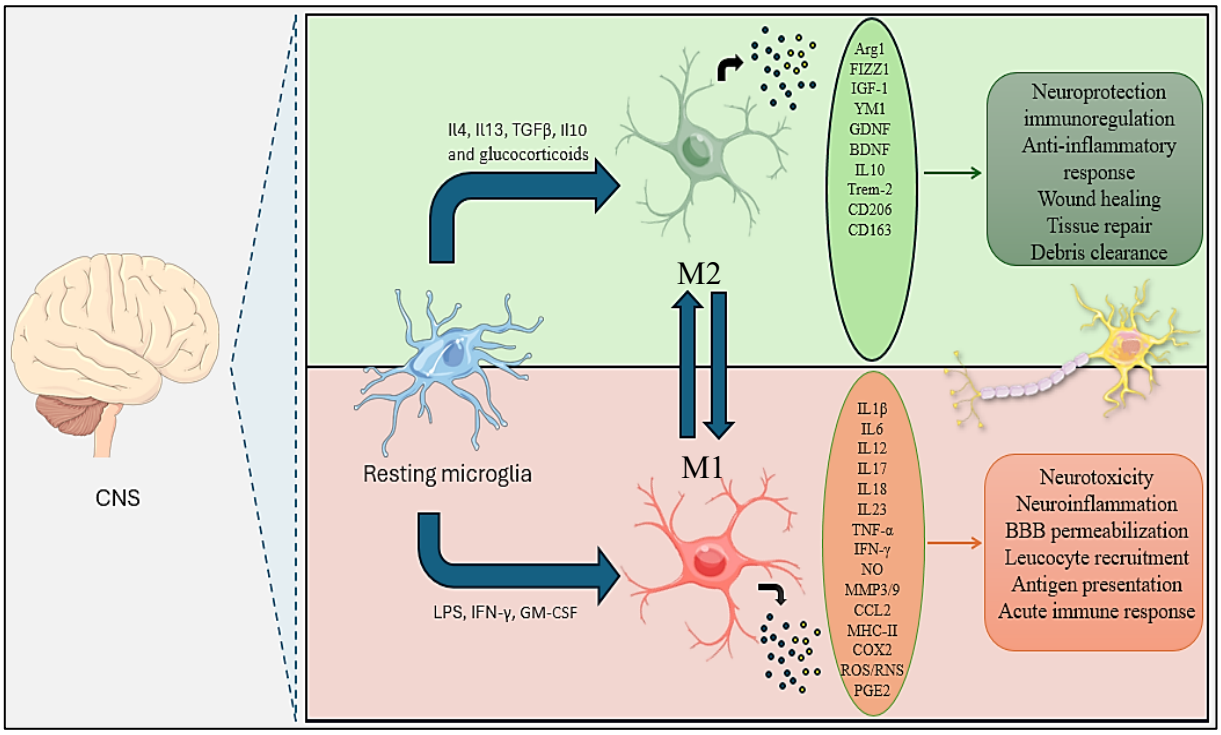

Figure 1

The alternative microglia activation phenotype: Presence of DAM in PD

Recent developments in single-cell RNA sequencing have revealed a range of microglial phenotypes linked to aging and Alzheimer's disease. Among these are "disease-associated microglia" (DAM), which are likely involved in protective phagocytosis, and "neurodegenerative microglia" (MGnD), which represent a malfunctioning microglial phenotype. Furthermore, proliferative-region-associated microglia (PAM) arise during development and express genes abundant in DAM. It is crucial to understand that the microenvironment can continuously affect the microglial phenotype in a manner that depends on time and context (64).

The etiology and activation of Disease-Associated Microglia (DAM) is primarily understood in the context of Alzheimer's Disease. In AD, DAM evolves through a two-step transition: from a homeostatic state to an intermediate Stage 1 and subsequently to Stage 2 DAM. Neurodegeneration-associated molecular patterns (NAMPs) potentially trigger the transition from the homeostatic state to Stage 1. Among the genes involved in initiating DAM, TREM2 seems to play a critical role in regulating the transition from Stage 1 to Stage 2 (65). Considering the resemblances between Parkinson's Disease and Alzheimer's Disease—both chronic, long-lasting conditions marked by the misfolding and accumulation of proteins—it seems reasonable to propose that the microglial subsets identified in AD may also be present in PD. Both neurodegenerative disorders progress over time, aligned with the buildup of misfolded proteins. While misfolded amyloid-β aggregates outside the cells, misfolded α-synuclein forms intracellular Lewy bodies; functionally, α-synuclein, similar to amyloid-β, creates a localized environment rich in NAMPs that promote the activation of microglia into a reactive state (66). Nevertheless, because most investigations model PD through short-term assessments, our comprehension of the long-term impacts of persistent α-synuclein NAMPs and microglial activation in PD is still limited. In other neurodegenerative conditions, where genes like APOE and TREM2 do not seem to influence disease risk or development, it is conceivable that different genes contribute to the emergence of a functionally comparable disease-associated microglial (DAM) response. In PD, genome-wide association studies and related evaluations indicate that P2RY12 (Purinergic Receptor P2Y, G-Protein Coupled, 12) might play a significant role in PD, likely involving its engagement in DAM activation (67).

Microglia in Parkinson's Disease

The activation of microglia starts early and

continues throughout the progression of Parkinson's disease. Reactive microglia were first observed in the substantia nigra of postmortem brain tissue from PD patients in 1988 (68). PD patients also exhibited increased expression of other markers of microglial activation, such as pro-inflammatory enzymes like iNOS and COX, as well as the phagocytosis-associated marker CD68. PET scans of PD patients revealed widespread microglial activation. Notably, microglial activation was found in individuals with long-term illnesses and newly diagnosed patients. Microglia in the SN of PD patients exhibited a higher proportion of amoeboid morphology, indicating a reactive state (69). Microglia possess pattern recognition receptors like TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and scavenger receptors (SRs) that allow them to recognize and respond to various stimuli, including extracellular α-synuclein. When α-syn accumulates outside of cells without proper clearance, it can activate microglia through PRRs, releasing inflammatory cytokines. The development of PD is influenced by chronic inflammation and neuronal damage. Interestingly, neuroinflammation caused by α-synuclein may occur even before the loss of dopaminergic neurons in PD (70, 71). The production of neuroinflammation might initiate α-synuclein oligomerization, creating a detrimental cycle of microglial activation. Further examinations of postmortem brain tissue from PD patients indicated that reactive microglia, characterized by amoeboid-shaped morphology, were linked to α-synuclein pathology in the SN and hippocampus. The protein α-syn is primarily located in presynaptic terminals and can be released from neurons through various mechanisms determined by its structure. Individual α-syn units are released passively when cell membranes are compromised, whereas aggregated forms of α-syn are released through non-traditional exocytosis or within multivesicular bodies (72, 73). As critical surveillance cells in the central nervous system, microglia are responsible for absorbing and breaking down α-syn. When internalized, α-syn can activate microglia, leading to neuroinflammation. In Parkinson's disease, different structural variants of α-syn can activate microglia, increasing the release of inflammatory molecules such as IL-6, IL-1β, and NO. Microglia have a significant capacity for phagocytosis, which is crucial for clearing α-syn and plays a role in developing Parkinson's disease. In Parkinson's disease, α-syn is overproduced and forms oligomers or protofibrils, which can propagate between cells and disrupt synaptic function. These formations also act as chemoattractants, guiding microglia toward damaged neurons. Excessive α-syn has been demonstrated to induce a pro-inflammatory state in microglia, leading to heightened production of inflammatory cytokines such as IL-1β, IL-6, and TNF-α, as well as enzymes like COX-2 and iNOS, and the generation of free radicals (74). The involvement of various receptors, including TLRs, TAM receptors (Tyro 3, Axl, and Mer), CD14 scavenger receptors, and TREM-2, is significant in the phagocytic activity of microglia. TLRs, specifically TLR2 and TLR4, are receptors found on microglia that play a role in the recognition and uptake of α-syn, ultimately enhancing its clearance by microglia (68). Oligomeric α-syn directly interacts with TLR1/2 and TLR4, inducing a pro-inflammatory M1 state (75). CD36 and P2X7 receptors are also implicated in the microglia activation by α-syn (76). The stimulated phagocytic activity of microglia is essential for clearing α-syn and preventing the accumulation of misfolded forms of α-syn. The extracellular form of alpha-synuclein is taken up by microglia, potentially via the autophagy receptor p62, and is degraded through selective autophagy (77). While microglia typically help clear misfolded protein aggregates, this process may adversely affect disease states. The internalization and processing of non-toxic α-syn by microglia can lead to the formation of disease-specific toxic forms through autophagy and impaired lysosomal breakdown. Multiple studies have highlighted the crucial role of the CX3CR1 receptor on microglia in mediating neuron-microglia communication through the CX3CL1 protein. The CX3CL1-CX3CR1 signaling pathway is essential for maintaining a healthy microglial activity balance, regulating chemoattraction and synaptic plasticity, and reducing microglia-induced inflammation and neurotoxicity (78, 79). Another key regulator of microglial activation is CD200, an immunoglobulin superfamily member found on neurons that interact with microglial CD200R. Disruption of the CD200-CD200R pathway has

been linked to increased microglial activation and degeneration of dopaminergic neurons (80). Furthermore, CB1 receptors are prevalent in neurons, while CB2 receptors are predominantly expressed in microglia in the brain. Several animal models of Parkinson's disease suggest activating microglial CB2 receptors can protect neurons and improve motor symptoms. Some genes associated with familial PD are involved in inflammatory processes (81). Mutations in the SNCA gene may cause abnormal conformation in α-synuclein, leading to increased conversion of soluble α-synuclein into insoluble aggregates found in PD. Exposure to extracellular α-synuclein can trigger the production of pro-inflammatory cytokines in microglial cells (82). Mutations in the LRRK2 (PARK8) gene are the most common genetic cause of both familial and sporadic PD. Previous research indicates high expression of LRRK2 in various immune cells, such as microglia, macrophages, and monocytes, while its expression in T cells is relatively low. This suggests that LRRK2 may primarily regulate PD's innate immune system and inflammation (83, 84). Studies on microglia have shown that activation of TLR2 or TLR4 can increase the expression and phosphorylation of LRRK2 (85). Researchers have identified specific chromatin regions in microglia that control LRRK2 expression and have demonstrated that a particular regulatory DNA element containing the PD-associated genetic variant rs6581593 modulates LRRK2 expression in microglia. These findings emphasize the importance of considering cell type when investigating the impact of non-coding genetic variants on disease pathogenesis and provide a mechanistic understanding of the relationship between the 5' region of LRRK2 and PD risk. In addition, inhibiting LRRK2 kinase activity can lead to the phosphorylation of NF-κB inhibitory subunit p50 at the protein kinase A (PKA)-specific phosphorylation site S337, resulting in a higher proportion of nuclear P-p50. This may hinder the function of NF-κB and impede efficient DNA binding and gene transcription activation in response to inflammation (86). The PARK7 gene encodes a small peptidase protein called DJ-1. DJ-1 dysfunction has been linked to a small proportion (1–2%) of inherited cases of early-onset PD (80). In microglia, reducing DJ-1 expression has been found to increase the production of inflammatory cytokines in response to LPS. Furthermore, DJ-1 may act as a scaffold protein, enabling the interaction between signal transducers and activators of transcription (STAT1) and its phosphatase, Src-homology 2-domain containing protein tyrosine phosphatase-1 (SHP-1), which negatively regulates the inflammatory responses of microglia. In DJ-1 knockout mice, microglia showed higher expression levels of phosphorylated STAT1 and inflammatory mediators COX-2, iNOS, and TNF-α. Additionally, microglia lacking DJ-1 exhibited increased mitochondrial activity, leading to elevated levels of ROS compared to normal microglia, which was further heightened by LPS treatment (87-89). Investigating genetic mutations associated with microglia significantly enhances our understanding of the cellular pathways implicated in PD. By uncovering these molecular mechanisms, we obtain valuable insights into the root causes and potential therapeutic targets by modulating specific genes to restore or modify microglial function.

Peripheral inflammation and Parkinson's disease

Evidence suggests that patients with Parkinson's disease have higher levels of pro-inflammatory cytokines and increased activation of circulating peripheral blood mononuclear cells. This indicates a link between PD pathology's peripheral immune and central nervous systems. Animal studies have also supported the relationship between neuronal degeneration and systemic inflammation. Reports suggest that inflammation in the body can lead to the loss of dopamine neurons in the brain. Clinical data supports the idea that inflammation may play a role in contributing to PD pathology, as PD patients with viral or bacterial infections exhibited significant motor and cognitive dysfunctions (90, 91). Peripheral inflammation can disrupt the blood-brain barrier, allowing inflammatory factors and immune cells to enter the central nervous system, which is a major contributing factor to PD development. However, it is still debated whether conditions that greatly recruit myeloid cells from the periphery are beneficial or detrimental to the disease. The specific role of peripheral immune activation versus recruitment and infiltration in this process still needs to be fully comprehended (92). It has been observed that monocytes and macrophages

can enter the inflamed brain. An increase in proteins associated with non-microglia myeloid cells, such as CD163, has been detected in the brains of individuals with Parkinson's disease. In rodent models of PD, a more significant presence of macrophages, particularly CD163+ macrophages, was also identified in the region of neurodegeneration (93, 94). The CCL2-CCR2 pathway has been implicated in the infiltration of monocytes into the inflamed brain. Studies in PD mouse models and patients have shown the upregulation and activation of CCR2, suggesting a detrimental role of infiltrating monocytes in PD. Additionally, differences in CCL2 levels in blood serum or cerebrospinal fluid have been associated with distinct clinical subtypes of PD (95). Overall, CD163+ and CCR2+ monocytes may contribute to neurodegeneration in PD through both peripheral actions and brain infiltration. PD patients have exhibited increased frequencies of Th1 cells and higher levels of IL10 and IL17A in their blood serum compared to healthy controls. However, microglial activation in the brains of PD patients did not show a significant correlation with peripheral inflammation markers. These findings suggest that peripheral adaptive immunity may indirectly influence microglial activation during the neurodegenerative process in PD (96).

Given the intricate causes and multifactorial aspects of Parkinson's disease, it is crucial to investigate the mechanisms of the disease and identify an optimal model. Over the last few decades, there have been significant discoveries and advancements in disease modeling, facilitated by various animal and cell models. Considerable progress has undoubtedly been made in the modeling of PD, and efforts continue to pursue a potentially ideal model that could lead to meaningful therapeutic successes.

Experimental Animal Models of PD

The use of animal models in Parkinson's disease research encompasses genetic and neurotoxin-based studies. In drug development, in vivo animal models are indispensable for pre-clinical trials, offering crucial insights into mutations, tissue pathology, motor symptoms, and behavioral patterns in various diseases. Although these models may not completely mirror neuropathological mechanisms, they have provided researchers with invaluable information about PD's progressive nature and even demonstrated substantial neurodegeneration in certain instances. Additionally, in vitro models involve primary microglia extracted from mice or rats, along with immortalized murine microglia cell lines like BV-2, which can be extensively cultured, thus aiding in reducing animals used in studies (97). Neurotoxins such as MPTP and 6-hydroxydopamine (6-OHDA) cause inflammation and subsequent degeneration in animal models. MPTP activates microglia and produces pro-inflammatory cytokines associated with M1, such as IL-6, IFNγ, and TNFα (98). Studies have suggested that the response of glial cells to MPTP reaches its peak before the death of dopaminergic neurons. Additionally, research has demonstrated that animals lacking IFNγ or TNFα do not exhibit susceptibility to MPTP-induced neurodegeneration (99). Separate research has observed the infiltration of T cells (CD4+) into the substantia nigra in MPTP-treated mice, and the reduction in dopaminergic neurons induced by MPTP was found to be lower in T cell-deficient mice, indicating the pro-inflammatory role of T cells (CD4+) in MPTP-induced neurotoxicity. Other studies have also revealed microglial activation in the substantia nigra through significant amoeboid morphological changes in the MPTP-induced Parkinson's disease model (100, 101). In a study conducted by Lee et al., it was observed that activation of the NLRP3 inflammasome in microglia derived from MPTP resulted in neuronal damage. Additionally, IL-1 receptor antagonists were found to exert protective effects against MPTP-induced neuronal damage (102). Shao et al. reported that TLR4 deficiency protects MPTP-induced PD mice by regulating motor impairment, DA neuronal damage, astrocyte/microglia activation, α-Syn, and NLRP3/NF-κB activation (103). Furthermore, Song et al. demonstrated that 2-hydroxy-4-methylbenzoic anhydride (HMA) reduced the activation of microglia and the expression of Iba-1, GFAP, and COX-2 in the striatum of MPTP-induced PD mice (104). Lastly, NBD-peptide-induced NF-κB inactivation was shown to cause downregulation of mRNA levels of GFAP, CD11b, iNOS, TNF-α, and IL-1β in the midbrain of MPTP-induced PD mice, as established by a recent result from Song et al (105). The 6-OHDA-induced PD model has demonstrated significant microglial activation through immunohistochemistry and PET imaging

(106). In a separate rat model induced by 6-OHDA, early microglial activation preceded the death of dopaminergic neurons, with phagocytic microglia observed earlier than those expressing the MHC II antigen. These observations strongly indicate the pathogenic involvement of microglia-mediated inflammation in PD (107). Neurotoxicity induced by 6-OHDA leads to an elevation in HSP60 levels. HMGB1 is associated with sterile inflammation in the brain, interacts with CD11b in microglia, and has been observed to bind to a-syn. Additionally, ATP release during cell death triggers microglial activation and chemotaxis response via purinergic receptors. Consequently, cell death has significant implications for microglial response, further influencing the immune response in the brain (108). In research utilizing knockout (KO) mouse models, scientists investigated the molecular mechanisms of IFN-γ and TNF-α. They observed that after MPTP treatment, IFN-γ KO mice displayed a fully restored phenotype, while TNF-α KO mice showed only minimal microglia activation. This finding suggested a synergistic interaction between the TNF-α and IFN-γ signaling pathways. Additionally, IFN-γ was found to play a substantial role in promoting neuronal cell loss after rotenone treatment, especially in the presence of microglia. Experiments using dopaminergic neuron-microglia co-cultures and rotenone treatment indicated that cultures with wild-type microglia, but not those with IFN-γ receptor-deficient microglia, exhibited a significant loss of dopaminergic neurons (109, 110). In rats, the combination of rotenone and lipopolysaccharide (LPS) was observed to induce neurodegeneration through microglia-mediated NADPH oxidase activation and release of ROS. Further investigation revealed that this NADPH activation directly results from HMGB1 release from microglia and dying neurons during ongoing LPS treatment, perpetuating the inflammatory loop (111). The accumulation of α-synuclein and the development of Lewy bodies and neurites containing α-synuclein have been identified as key features of PD pathology since the first examination of SNpc tissue using immunohistochemistry. Duplication, triplication, and specific mutations (A30P, A53T, E46K, H50Q, and G51D) in the SNCA gene lead to an inherited form of PD. Mouse models of SNCA include treatments with α-synuclein, excessive expression of human α-synuclein, and transgenic models incorporating specific human α-synuclein mutations. The aggregation and harmful effects of α-synuclein are subsequent events following oxidative stress, genetic changes, and post-translational modifications such as phosphorylation, nitration, ubiquitination, etc (112). In transgenic strains experiencing dopaminergic neuronal death, such as those with the a-syn A53T mutation under the prion protein promoter (PrP) or the double mutant (DM) A30P+A53T a-syn under the TH promoter, there were observations of microgliosis and alterations in the expression of multiple immune-related genes preceding the cell death. Additionally, mice with overexpressed a-syn under the TH promoter exhibited early microgliosis and increased TNF-α levels despite the absence of cell death. Similarly, microgliosis in the substantia nigra and striatum of the Thy-1 wild type a-syn line occurred before motor deficits and regardless of the absence of neuronal death (113, 114). Several genes associated with Parkinson's disease have been investigated for their role in the immune response of microglia and the release of pro-inflammatory cytokines. Transgenic animal models related to different causative genes in neuroinflammation-mediated PD have been employed to study these processes. In a murine model, suppression of the PRKN gene resulted in elevated GSH levels, decreased astrocyte proliferation, and increased microglial proliferation (115). Another study using a knockout mutation of PRKN and low LPS treatment over an extended period demonstrated that a deficiency of PRKN expression leads to increased susceptibility to dopaminergic degeneration in the substantia nigra. Additionally, aging in PRKN knockout mice led to abnormal elevation in midbrain microglial activation (116). In a rat model, the knockout mutation of LRRK2 showed resistance to dopaminergic neuron degeneration, which was associated with decreased numbers of pro-inflammatory microglia in the SN (117). Brain slices from PINK1-knockout mice confirmed an increase in the expression levels of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα (118). DJ-1−/− mice treated with LPS exhibited high expression of CD14, and reduced numbers of dopaminergic neurons were detected in the SN via tyrosine hydroxylase (TH) staining (87).

Due to the complex nature of experimental technology, developing Non-Human Primate (NHP) animal models requires substantial financial and labor investments. Additionally, ethical considerations limit their application, resulting in fewer studies utilizing NHP models. Nevertheless, unlike other model organisms, NHPs provide a more accurate and comprehensive simulation of human Parkinson's Disease symptoms, offering enhanced opportunities for investigating clinical treatment options. Given their close evolutionary relationship with humans, NHPs exhibit substantial genetic, physiological, and behavioral diversity that parallels human beings (119). As a result, findings from studies on cognition and behavior related to PD in NHP models tend to offer more reliable insights into human PD. MPTP-induced NHP models have been extensively employed in preclinical research, with older monkeys demonstrating greater susceptibility to infection than younger ones. However, neurotoxin-induced models often present a relatively unstable phenotype, complicating the replication of PD's gradual progression. To address this challenge, some researchers have employed stereotaxic injection of MPP+ into specific regions of the unilateral substantia nigra pars compacta (SNpc), guided by magnetic resonance imaging. Follow-up experiments have detected α-synuclein in the MPTP-induced monkey model of PD, potentially enhancing the development of PD animal models. Furthermore, the expression of PINK1 has been observed to be higher in NHPs than in mouse models, suggesting that PINK1 may play a more significant role in NHPs than in the mouse model (97).

Microglial-targeted therapies in PD

Activation of microglia via the M1 phenotype leads to an output that is both pro-inflammatory and pro-killing. It is possible to target its downstream signaling pathways to reduce the inflammatory damage caused by M1 activation of microglia. IFN-γ triggers the M1 phenotype through the JAK/STAT signaling pathway; inhibiting this pathway may halt M1 activation. Indeed, research indicates that blocking the JAK/STAT pathway results in the suppression of downstream genes associated with the M1 phenotype in various disease models (120). Another strategy to mitigate M1 activation is to focus on pro-inflammatory cytokines like TNF-α, IL-1β, and IFN-γ, limiting their ability to engage with receptors on other cell types. TNF has been addressed through various strategies in PD animal models to reduce M1-related toxicity. A single injection of lentivirus carrying dominant-negative TNF (DN-TNF) into the substantia nigra of rats, alongside a 6-OHDA lesion in the striatum, diminishes the loss of dopaminergic neurons and associated behavioral issues in these rats (121). In another study focusing on TNF's role in a delayed and progressive neurodegeneration model, rats that received DN-TNF in the substantia nigra two weeks post-6-OHDA lesion, exhibited no further loss of dopaminergic neurons, even after five weeks of 6-OHDA treatment, indicating TNF's critical role in inflammation and its potential as a therapeutic target in PD (122). Peroxisome proliferator-activated receptors (PPARs) play a significant role in activating microglia and inflammatory pathways. Agonists of PPARs are believed to be beneficial for treating PD and other neurodegenerative conditions. The treatment with the PPARγ agonist, rosiglitazone, halts degeneration in both the striatum and substantia nigra pars compacta (SNpc) by reducing TNF-α production and altering microglial polarization in the MPTPp (MPTP + probenecid) progressive mouse PD model (123). Pioglitazone, another PPARγ agonist, protects against the loss of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra and partially prevents dopamine (DA) decrease in the striatum of MPTP-treated mice. Treatment with pioglitazone leads to reduced microglial activation, lower iNOS production, and decreased nitric oxide-related toxicity in both the striatum and substantia nigra. Tanshinone I, a natural flavonoid, diminishes the generation of M1 pro-inflammatory mediators (nitric oxide, TNF-α, IL-6, and IL-1β) while inhibiting the expression of G-CSF and NF-κB following LPS-induced inflammation in BV2 microglial cell lines. In the MPTP model for PD, Tanshinone I prevents neurotoxicity in dopaminergic neurons enhances motor function and stabilizes striatal neurotransmitter levels. Piperine, a naturally occurring bioactive compound, alleviates the loss of TH-positive neurons in the substantia nigra and the motor deficits induced by MPTP. Additionally, piperine reduces MPTP-induced microglial activation, lowers pro-inflammatory IL-1β expression, and

limits apoptosis in these mice (124). The molecules capable of activating the anti-inflammatory M2 phenotype or facilitating the transition from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype may hold therapeutic potential for Parkinson's disease. Anti-inflammatory molecules such as IL-10 and beta interferons confer neuroprotection by modulating the balance between M1 and M2 phenotypes. In an MPTP mouse model of PD, the cerebral infusion of AAV-expressing human IL-10 results in decreased expression of the pro-inflammatory iNOS and significantly enhances levels of anti-inflammatory mediators, including IFN-γ and transforming growth factor-β. This infusion also prevents the loss of striatal dopamine and reduces transcriptome levels of tyrosine hydroxylase, indicating a neuroprotective effect in MPTP-intoxicated mice (125, 126). Additionally, the endocannabinoid system has been implicated in the pathogenesis of PD (127). In a chronic MPTPp model of PD, administration of an inhibitor that prevents the degradation of 2-AG—an endocannabinoid ligand—ameliorates MPTPp-induced motor impairment and protects the nigrostriatal pathway (128). MPTPp mice treated with this inhibitor demonstrate beneficial microglial phenotypic changes and restorative microglial activation alongside increased levels of TGF-β and GDNF. Furthermore, dimethyl fumarate (DMF), an approved treatment for multiple sclerosis, protects against the depletion of striatal DA and its transporters while reducing the MPTP-induced increase in IL-1β and COX-2 activity in an MPTP mouse model of PD. DMF also modulates microglial activation states and restores levels of nerve growth factor, providing neuroprotection in MPTP-intoxicated mice (124).

CONCLUSION

The intricate and diverse etiology of Parkinson's disease poses a significant challenge to developing effective treatments. There is substantial evidence linking inflammation to the progression of PD. Recent advancements in the field have reshaped our understanding of PD as a complex chronic inflammatory disorder. Numerous studies indicate that the pathways of α-synuclein accumulation and inflammation may intersect and exacerbate the progression of PD. Despite recent progress, the molecular and cellular mechanisms driving PD progression remain incompletely elucidated. A more comprehensive understanding of these mechanisms will improve our grasp of PD pathology and also aid in identifying novel therapeutic prospects. In recent years, there has been a significant increase in our understanding of the role of microglia in neurodegenerative diseases. Through advanced techniques such as single-cell RNA sequencing (scRNA-seq) and single-nucleus RNA sequencing (snRNA-seq), we have been able to identify gene expression signatures of microglia at the individual cell level. This has revealed that specific microglial states are associated with pathological characteristics and distinct functions. Microglia contribute to the progression of neurodegenerative diseases through various mechanisms, including impaired removal and propagation of pathological deposits, neuroinflammation, and altered microglial phenotypes, ultimately leading to disease progression and neurodegeneration. The infiltration of peripheral immune cells can induce a pro-inflammatory phenotype in microglia, accelerating disease progression. Dysfunctional microglia can also impact neuronal plasticity and activity by facilitating the elimination of synapses and perineuronal nets. It is important to note that microglia have beneficial and detrimental effects on neurodegenerative diseases. While significant progress has been made in understanding the functions of microglia, there is still a need for further clarification on their heterogeneity and dynamics during disease progression. The recent introduction of high throughput omics data analysis has been instrumental in identifying and elucidating the functional roles of microglia in specific states. Additionally, this advancement has helped uncover dysregulated pathways and critical molecules crucial to disease pathogenesis, which will aid in developing new therapeutic approaches. The gradual degeneration of dopaminergic neurons is an unavoidable natural process associated with aging, and it is difficult to comprehend that microglia mainly exacerbate damage to neurons and neural circuits. Emphasizing the positive impact of microglia on maintaining balance in brain functions could lead to developing new treatment options for Parkinson's disease. Current neurotoxin-induced animal models of Parkinson's disease come with

significant limitations that hinder our understanding of this complex condition. While these models are designed to mimic some aspects of PD, they often trigger only limited biochemical responses and fail to capture the hallmark pathological features seen in humans. Moreover, the highly toxic substances used in these models lead to irreversible damage to the animals' nervous systems, making it challenging to evaluate the efficacy of potential PD treatments effectively. This reliance on exogenous neurotoxin models has contributed to the high failure rate of numerous clinical drug trials. These models promote the rapid destruction of dopaminergic neurons, which starkly contrasts with the gradual progression of idiopathic PD in humans. As a result, forward-thinking researchers are pushing for a shift toward using endogenous neurotoxins to develop models that mimic the slow-onset symptoms characteristic of idiopathic PD. This strategic pivot opens the door for more relevant and robust preclinical studies. Among the potential actors in this new wave of PD research are non-human primates, whose close genetic and physiological ties to humans make them a promising choice. These extraordinary models can accurately reproduce the clinical symptoms of PD, providing invaluable insights into the underlying pathological processes and the effectiveness of therapeutic drugs. Embracing NHP models marks a significant step toward refining our approach to Parkinson's research. In addition to NHPs, innovative models like the MitoPark mouse model, α-syn PFF models, iPSC-based models, optogenetic models, and gene editing models are emerging, each promising to enrich our understanding of PD. As biomedical technology advances, the dream of creating the ideal animal model for PD seems increasingly attainable. Researchers are diligently refining and enhancing existing models to reflect the traits and progression of PD in humans. These efforts inspire hope that we will soon unlock the secrets of Parkinson's disease, ultimately paving the way for breakthroughs that can benefit countless lives.

ETHICAL CONSIDERATIONS

Compliance with ethical guidelines

This study is a narrative review without involving humans or experimental animals.

ACKNOWLEDGMENT

There is nothing to say.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

FUNDING

This study was conducted without any external financial support.

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