MICROGLIA IN HEALTH AND DISEASE: A UNIQUE IMMUNE CELL POPULATION

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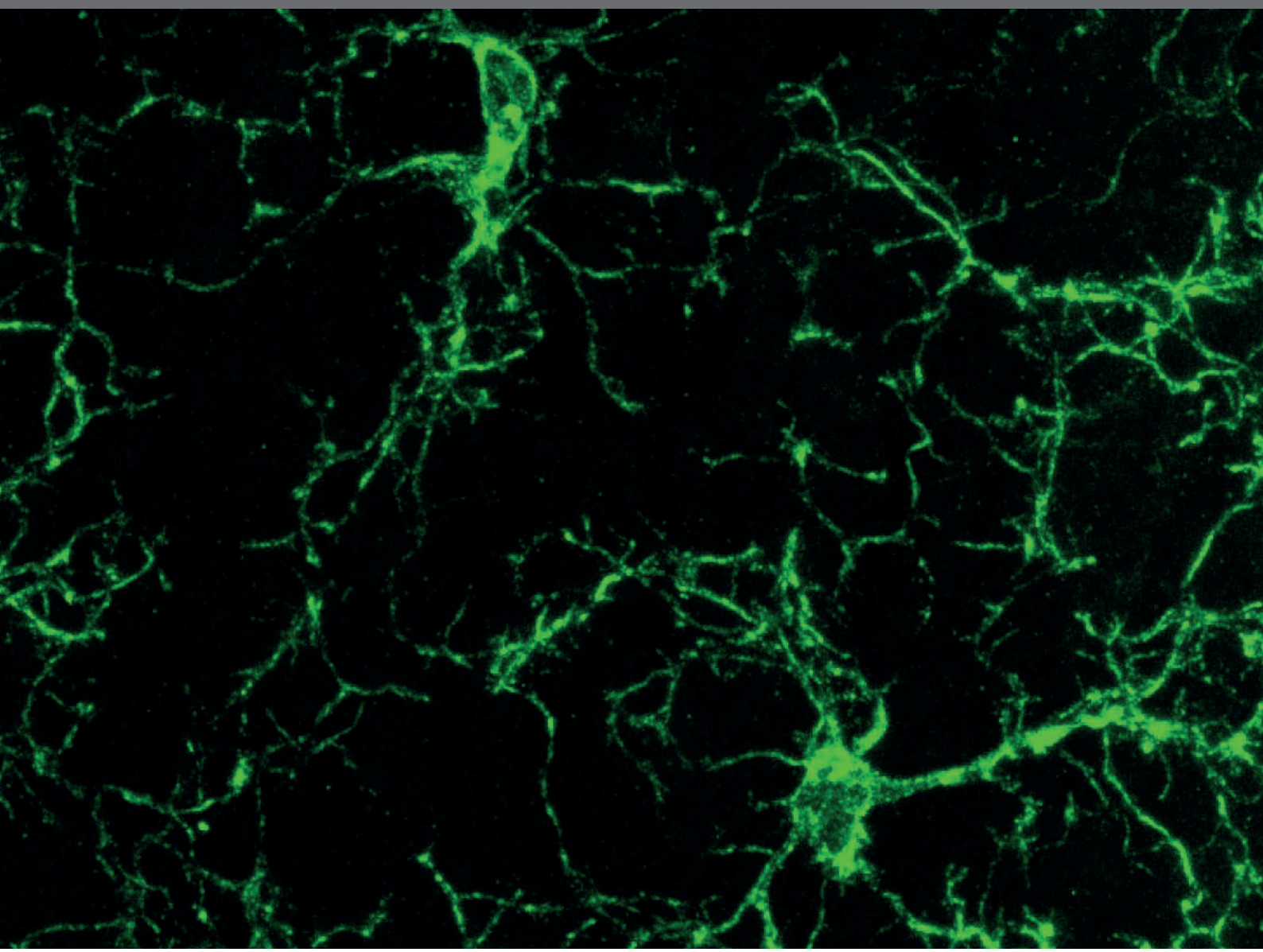

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MICROGLIA IN HEALTH AND DISEASE: A UNIQUE IMMUNE CELL POPULATION

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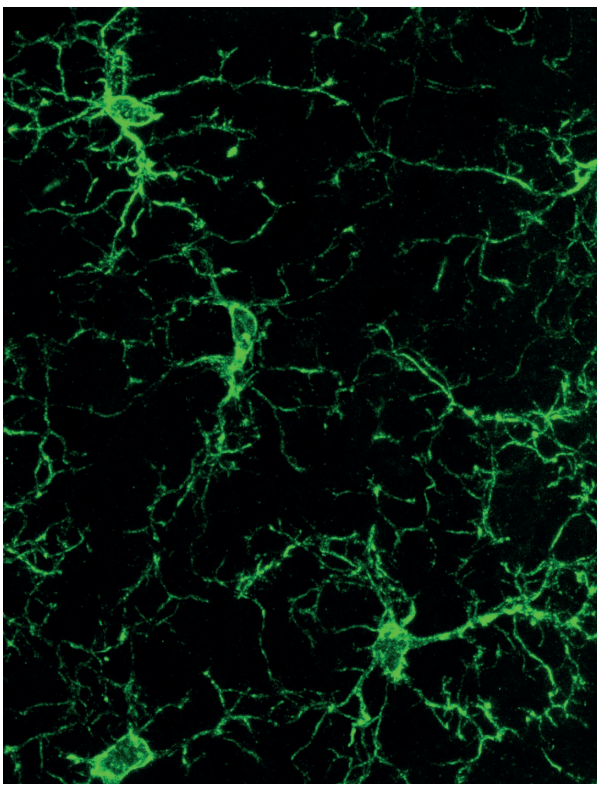

Brain murine microglia stained for IBA1 (green).
Image: Carole Sousa.

Microglia are essential for the development and function of the adult brain. Their ontogeny, together with the absence of turnover from the periphery and the singular environment of the central nervous system (CNS), make microglia a unique cell population compared to other tissue-macrophages. The unique properties and functions of microglial cells, such as their role in synaptic pruning or the exceptional capacity to scan the brain parenchyma and rapidly react to its perturbations, have emerged in recent years. In the coming years, understanding how microglia acquire and maintain their unique profiles in order to fulfil distinct tasks in the healthy CNS and how these are altered in disease, will be essential to develop strategies to diagnose or treat CNS disorders with an immunological component.

This Research Topic covers several aspects of microglial biology, ranging from their origin and the functional role of microglia during development and lifespan, their molecular properties compared with other brain and peripheral immune cells to microglial phenotypes and functional states in neurodegenerative diseases and brain tumours. In conclusion, the present Research Topic provides a comprehensive overview of our current understanding of several cellular and molecular mechanisms that make microglia a unique immune cell population within the healthy CNS as well as under inflammatory, neurodegenerative and tumorigenic processes.

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EDITORIAL
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Microglia in Health and Disease: A Unique Immune Cell Population

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Editorial on the Research Topic

Microglia in Health and Disease: A Unique Immune Cell Population

Microglia are essential for the development and function of the adult brain. Their ontogeny, together with the absence of turnover from the periphery and the singular environment of the central nervous system (CNS), makes microglia a unique cell population compared with other tissue macrophages. Supporting this notion, recent transcriptional studies have revealed that microglia display specific gene expression signatures that are clearly distinct from other brain and peripheral immune cell populations in the healthy and diseased CNS (1).

The unique properties and functions of microglial cells, such as their role in synaptic pruning or the exceptional capacity to scan the brain parenchyma and rapidly react to its perturbations, have emerged in recent years. In the coming years, understanding (i) how microglia acquire and maintain their unique profiles to fulfill distinct tasks in the healthy CNS and (ii) how these are altered in disease, will be essential to develop strategies to diagnose or treat CNS disorders with an immunological component.

In this research topic, four original articles and four reviews cover several aspects of microglial biology, ranging from their origin and the functional role of microglia during development and lifespan, their molecular properties compared with other brain and peripheral immune cells to microglial phenotypes and functional states in neurodegenerative diseases and brain tumors.

In particular, Menassa and Gomez-Nicola describe the various historical schools of thought that had debated the origin of human microglial cells. The data that have recently been accumulated on microglial dynamics in the developing human brain, together with the evidence obtained from rodent studies on the functional role of microglia during development, allowed them to identify limitations for the existing approaches in human studies.

Savage et al. and colleagues focus on defining the distinctive ultrastructural features of microglia and the unique insights into their function that can be provided by electron microscopy. These techniques will further revolutionize the study of microglia across lifespan, brain regions, and contexts of health and disease.

Ayana et al. conducted a meta-analysis of brain cells, using publicly available datasets, for deciphering microglia-specific expression profiles, thus identifying putative novel human microglia markers across lifespan and different brain regions.

In her review, Sevenich summarizes recent developments in evaluating the distinct functions of brain-resident and recruited myeloid cells in neurodegenerative disorders and brain cancers, thus underlying disease- and cell-type-specific effector functions of microglia and macrophages.

Walentynowicz et al. attempt to study glioma-associated microglia/macrophage functional states by identifying marker genes from different experimental models and clinical samples, revealing only a small set of common genes, thus reflecting divergent responses depending on specific clinical and experimental settings.

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A study by Blank et al. suggests that early retinal microglia activation represents a first step in the pathological cascade of retinitis pigmentosa, which might initiate or accelerate photoreceptor degeneration, thus pointing out that activated microglial cells may be responsible for the onset or amplification of the degenerative process.

In this line, Zhou et al. show that the severity of retinal degeneration is related to microglia polarization. Specifically, the authors revealed that alpha-1 antitrypsin, a novel immunomodulatory agent in autoimmune diseases and transplantation, shifts microglia toward an anti-inflammatory phenotype along with a protective effect on retinal degeneration.

Finally, Kounatidis and Chtarbanova review the recent findings obtained in the fruit fly (*Drosophila melanogaster*) on the contribution of glial innate immune pathways in lifespan and neurodegeneration. In particular, the NF-κB pathway and the phagocytic ability appear to be major contributors to lifespan, possibly through an immune–neuroendocrine axis.

In conclusion, this research topic provides a comprehensive overview of our current understanding of several cellular and molecular mechanisms that make microglia a unique immune cell population within the healthy CNS as well as under inflammatory, neurodegenerative, and tumorigenic processes. Furthermore, an emerging key aspect of microglia is their heterogeneity across the CNS under homeostatic or pathological conditions. Established methodologies combining single-cell approaches, such as single-cell RNA sequencing, with functional screening of inferred cellular diversity, are starting to reveal microglia diversity. The application of single-cell techniques to phenotype homeostatic and activated microglia under different neurological disorders are currently opening new avenues to understand cellular and functional states of various subpopulations in a context-dependent manner, thus shedding more light on microglia unique features.

AUTHOR CONTRIBUTIONS

AM wrote the manuscript. All the authors edited and approved the manuscript.

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1. Sousa C, Biber K, Michelucci A. Cellular and molecular characterization of microglia: A unique immune cell population. *Front. Immunol* (2017) 8:198. doi:10.3389/fimmu.2017.00198

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MINI REVIEW
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Microglial Dynamics During Human Brain Development

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Microglial cells are thought to colonize the human cerebrum between the 4th and 24th gestational weeks. Rodent studies have demonstrated that these cells originate from yolk sac progenitors though it is not clear whether this directly pertains to human development. Our understanding of microglial cell dynamics in the developing human brain comes mostly from postmortem studies demonstrating that the beginning of microglial colonization precedes the appearance of the vasculature, the blood–brain barrier, astrogliogenesis, oligodendrogenesis, neurogenesis, migration, and myelination of the various brain areas. Furthermore, migrating microglial populations cluster by morphology and express differential markers within the developing brain and according to developmental age. With the advent of novel technologies such as RNA-sequencing in fresh human tissue, we are beginning to identify the molecular features of the adult microglial signature. However, this is may not extend to the much more dynamic and rapidly changing antenatal microglial population and this is further complicated by the scarcity of tissue resources. In this brief review, we first describe the various historic schools of thought that had debated the origin of microglial cells while examining the evidence supporting the various theories. We then proceed to examine the evidence we have accumulated on microglial dynamics in the developing human brain, present evidence from rodent studies on the functional role of microglia during development and finally identify limitations for the used approaches in human studies and highlight under investigated questions.

Keywords: microglia, brain development, glial cells, proliferation, human brain, neurodevelopment

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INTRODUCTION

Microglia are resident macrophages of the mammalian central nervous system (CNS) with a plethora of functions including innate immunity and neuroprotection (1, 2), synaptic pruning (3), and the phagocytosis of cellular debris (4). Microglia also have a dark side, suggested to be indirectly involved in pathologies such as schizophrenia (5) and more directly in Alzheimer's disease (6). They play a central role in driving neuroinflammation and also coordinating this response with the systemic compartment and with other glial cells including astrocytes (7). A careful characterization of human microglial morphology, transcriptome, regional heterogeneity, and turnover in the adult human brain is emerging greatly due to the increased availability of appropriately preserved postmortem tissue. In the adult human cortex, for example, microglial cells acquire a ramified morphology with processes surveying the neighboring environment and an amoeboid morphology with retracted processes when they are phagocytosing the debris of dying neurons or synapses or reacting to a foreign body (8). Adult human microglia are enriched for genes associated with the

TABLE 1 | A summary of main microglial topography studies in embryonic and fetal human tissue.

Reference	Year	Age (weeks)	Sample size	Anatomical areas	Markers used	Findings and conclusions
Rydberg (24)	1932	38.0	2	Cerebrum, cerebellum, spinal cord	Silver carbonate	First study on microglial cytogenesis in human fetuses and newborns. Proponent of the neuroectodermal theory, this study suggests that any glial cell could originate from amoeboid glia located along the ependymal matrix
Von Santha (23)	1932	24.0 and 29.0	2	Basal ganglia, telencephalon, white matter tracts, subcortex, medulla	Silver carbonate	Microglia are ramified in the basal ganglia, the telencephalon, and the medulla. Plump branched cells are observed in the subcortex and prospective white matter tracts. No globose forms are reported
Juba (25)	1933	10.0–34.0	7	Cerebrum, cerebellum	Silver carbonate	Microglia are amoeboid early in development and ramify when they become differentiated. Microglia enter the developing brain from mesodermal elements closely associated with the vasculature. Microglia are genetically related to monocytes
Kershman (76)	1939	8.0–29.0	22	Cerebrum, cerebellum, spinal cord	Silver carbonate, H&E	The youngest form of microglia is amoeboid first before they ramify into a mature form. Amoeboid cells enter into the brain from specific locations known as fountains located around the choroid plexus, the meningeal compartment, and blood vessels. First evidence of microglia is in the rhombencephalon at 8.0 weeks. Once in the developing brain, amoeboid microglia acquire pseudopods as they penetrate deep into the brain matter and eventually acquire a final stable ramified phenotype
Choi (62)	1981	6.0–22.0	53	Cerebrum, cerebellum, spinal cord	H&E; Toluidine Blue	6.0–12.0 weeks: hematogenous cells are found in the cerebellum, spinal cord, and cerebrum. These become rarer as the fetus gets older. The neuropils of embryos/fetuses contain macrophage-like cells inside and outside of blood vessels. Subpial, perivascular, and perineuronal regions of neural parenchyma contain small cells with fusiform nuclei, some with elongated nuclei and some with rod-shaped/oblong nuclei
Fujimoto et al. (66)	1989	7.5–26.0	7	Cerebrum	NDP-ase	Vascularization in the cerebral cortex is apparent by 9.0 weeks, vessels invade the pallium. Amoeboid NDP-ase cells appear around the vessels. The MZ has poorly ramified microglia. By 11.0 weeks, amoeboid cells present in the matrix and subcortical layers. Highly ramified NDP-ase cells are seen in layer I, the subcortex, and cortical mantle between 22.0 and 26.0 weeks
Hutchins et al. (77)	1990	13.0–24.0	22	Frontal cerebrum	RCA-1; EBM-11	Densest RCA-1 and EBM-11 cells are present in the germinal matrix at 13.0 weeks. Microglial morphology is variable between the gray and white matter between 13.0 and 18.0 weeks (amoeboid versus intermediate) and less variable and ramified across the mantle and white matter between 19.0 and 24.0 weeks
Esiri et al. (86)	1991	18.0-term	15	Telencephalon	EBM-11; HLA-DR	Amoeboid cells are present in the germinal matrix at high densities throughout this temporal window. Ramified microglia in gray and white matter are only seen from 35.0 weeks. Very little reactivity to HLA-DR is detected throughout
Gould and Howard (80)	1991	13.0–27.0	19	Cerebral white matter	A1ACT; MAC387	Macrophage clusters positive for A1ACT accumulate in prospective white matter hotspots including the periventricular areas, corpus callosum, the junction between the external and internal capsule, the optic tract, and the thalamus. Increased densities followed a caudo-rostral pattern with more rostral areas showing microglial fountains after 22.0 weeks
Hutchins et al. (90)	1992	18.0–24.0	6	Cervical spinal cord	RCA-1; EBM-11	RCA-1/CD68-positive amoeboid microglia are seen in the white matter and only RCA-1 but CD68 negative ramified microglia are seen in the gray matter
Wierzba-Bobrowicz et al. (43)	1995	7.0-term	47	Mesencephalon	RCA-1; Anti-Ferritin	RCA-1/Ferritin-positive amoeboid cells are observed in the mesencephalon between 16.0 and 40.0 weeks

(*Continued*)

TABLE 1 | Continued

Reference	Year	Age (weeks)	Sample size	Anatomical areas	Markers used	Findings and conclusions
Rezaie et al. (82)	1997	16.0–22.0	7	Frontal telencephalon	CD11b; CD68; CD64; CD45; RCA-1; BSB-4	Fetal RCA1 microglia co-occur with highly vascularized ICAM-1 positive vessels within the developing brain
Andjelkovic et al. (63)	1998	4.5–13.5	14	Telencephalon, diencephalon, rhombencephalon	RCA-1; Tomato Lectin; CD68	At 5.5 weeks, CD68 and lectin positive cells are observed in the cerebellum and medulla. Between 6.0 and 7.5 weeks, first blood vessels in telencephalic wall and CD68/lectin cells increase in density in line with vascularization. Vascularization by CD34 tagging is prominent first caudally (cerebellum and diencephalon), then rostrally eventually. At 7.5 weeks, ramified microglia are detected in the cerebellum only among amoeboid cells. Between 8.0 and 9.0 weeks, clusters of microglia are observed in diencephalon. Ramified microglia are also observed in the MZ and IZ and none in CP. By 11.0–13.0 weeks, ramified microglia are detected in all layers of the cortex. Two classes of cells are identified here lectin positive and CD68/lectin positive
Wierzba-Bobrowicz et al. (12)	1998	8.0–22.0	72	Mesencephalon, cerebellum, frontal lobe	RCA-1; Anti-Ferritin; HAM56	Amoeboid microglia are increased in all structures as gestation progresses. White matter ramified microglia are highest in density by 22 weeks in the mesencephalon and the lowest in the white matter of the cerebellum
Rezaie et al. (31)	1999	7.0–15.0	9	Spinal cord	RCA-1; CD68; HAM56; CD11b; CD45; CD64	Microglia present are present in the spinal cord from 9.0 weeks and enter from the meninges/connective tissue unlike the cerebrum where they migrate out from the germinal matrix toward the cortical surface
Rezaie et al. (75)	2005	12.0–24.0	45	Telencephalon	RCA-1; CD68; MHC-II	12.0–14.0 weeks of gestation, 2 populations are detected: CD68+/RCA-1+/MHC-II– amoeboid cells aligned within the subplate and RCA-1+/CD68–/MHC-II- cells in the MZ and lower CP that progressively ramify within the subplate. Microglia are absent from the germinal layers and IZ. From 14.0 to 15.0 weeks, MHC-II+/CD68+ cells in the germinal layers and corpus callosum appear and further populate the lower telencephalon from 18.0 to 24.0 weeks
Billiards et al. (84)	2006	22.0–37.0	9	Cerebral white matter	Tomato lectin; CD68; MHC-II	Ramified microglia are only detected in the prospective white matter at 22.0 weeks then intermediate or amoeboid forms are seen between 25.0 and 37.0 weeks in similar areas
Monier et al. (60)	2006	5.0–24.0	31	Diencephalon, telencephalon	IBA-1; CD68; CD45; MHC-II; LN3; HLA-DR	By 5.5 weeks, accumulation of IBA-1 microglia near the meninges and choroid plexus before blood vessel formation in the brain parenchyma. Early entry along the di-telencephalic fissure of amoeboid cells from within the choroid plexus and meninges is reported. These are fetal macrophages reported by Choi and Andjelkovic et al. (1998). IBA-1/CD34 associated cells are observed at 10.0 weeks in the white matter
Monier et al. (61)	2007	4.5–24.0	33	Telencephalon	IBA-1; CD68; CD45; MHC-II; LN3; HLA-DR	Microglia express IBA-1 at 4.5 weeks. Amoeboid cells are present in the lumen and leptomeninges then. Entry into the brain is through the ventricles, whereby microglia assume eventually tangential and radial migration routes. Other route is from the pia to the MZ. Microglia are ramified by 22.0 weeks in the cortical mantle, amoeboid in the IZ, and grouped in clusters between 22.0 and 23.5 weeks
Verney et al. (73)	2010	20.0–32.0	4	Telencephalon, diencephalon	IBA-1	Accumulation of IBA-1 microglia in clusters is seen in the white matter (centrum semiovale)

(*Continued*)

TABLE 1 | Continued

Reference	Year	Age (weeks)	Sample size	Anatomical areas	Markers used	Findings and conclusions
Cho et al. (89)	2013	15.0–25.0	9	Brainstem, olive, gray matter, white matter, hippocampus	H&E; HLA-DR2; CD68	CD68 positive cells are evident in the floor of the fourth ventricle, the pons and olive at 15.0–16.0 weeks, accumulating in and around the hippocampus at 22.0–25.0 weeks. At both stages, the accumulation of these cells was evident in the optic tract and the anterior limb of the internal capsule. GAP-43 developing axons are not associated with CD68 cells
Mildner et al. (88)	2016	11.0–35.0	4	Cerebellum, white matter, cortex	P2Y12; CD68; IBA-1; HLA-DR	Clusters of microglia are detected between the lateral ganglionic eminence and the caudate nucleus, as well as in nerve fibers spanning the caudoputamen. Two smaller clusters of P2Y12 microglia are also visible in the thalamic region and the optic tract. All P2Y12 microglia express IBA-1. Non-clustered microglia have moderate CD68 immunoreactivity but strong P2Y12 reactivity. P2Y12 amoeboid myeloid cells are seen in the choroid plexus in an 11.0-week fetus. Choroid plexus cells are positive for IBA-1, but show very low immunoreactivity against P2Y12

CP, cortical plate; IZ, intermediate zone; H&E, hematoxylin and eosin; MZ, marginal zone.

inflammasome, cell-adhesion, and immune modulation among others and these characteristic profiles have been helpful in defining the differences and similarities with the better characterized model of murine microglia (9, 10). Adult human microglia also demonstrate regional heterogeneity with lower densities in the gray matter compared with the white matter and substantially lower densities in the cerebellar cortex, for example, compared to regions with higher densities such as the substantia nigra (11, 12). Adult human microglia renew slowly at a median rate of 28% per year and at any given moment, only 2% of microglia are thought to be proliferating (13, 14). It is suggested that the spatiotemporal coupling between proliferation and apoptosis maintains microglial density throughout life (14). In sum, though our understanding of the human adult microglial population has progressed tremendously, we know very little about these cells during human brain development. In this short review, we first present a brief history of the discovery of microglia and then examine the various schools of thought that had debated the origins of these cells. We then proceed to present an overview of what is known about microglia during human brain development (see **Table 1** for a summary of the main studies on microglia in developing human tissue) and examine how rodent studies have helped elucidate some of the key functions of microglia during brain development. We conclude by discussing the challenges associated with the utilized approaches in human studies.

ON THE DISCOVERY OF MICROGLIA

In 1899, Franz Nissl, a German neuropathologist, reported on the presence of rod-shaped cells in human brain cases of syphilitic paralysis, a severe neuropsychiatric condition characterized by eventual dramatic cerebral atrophy (15). This was perhaps one of the earliest reported descriptions of rod-shaped/bipolar microglia, thought to correspond to an intermediate phenotype of cells. Decades later, Kreutzberg reported on the response of microglial cells to injury in a rodent model of facial nerve axotomy (16). Unlike Nissl's more general observation, Kreutzberg was specifically investigating how the microglial population reacted to injury and demonstrated a marked increase in proliferation in rodents. Mononuclear phagocytes of the CNS such as microglia and macrophages were known as the mesoglia, being of mesodermal origin, and this classification distinguished them from astrocytes and oligodendrocytes that are of neuroectodermal origin (17). In 1913, Cajal optimized Camillo Golgi's black reaction method by using the gold chloride sublimate to enhance the visualization of non-neuronal elements of the CNS that the first morphological description of the microglial cell body emerged (18). Cajal identified cell bodies without processes differentiating them from neurons and astrocytes. Due to limitations in his method, he could not observe the ramifications emanating from microglial cells. Subsequently, Pio Del Rio Hortega optimized Cajal's histochemical method and used silver carbonate to selectively label and visualize the cell body and the ramified morphology of these cells (19). He also incepted the term "microglia" in 1919 and provided a thorough morphological and functional characterization of these cells from development to injury, providing seminal findings that settled long lasting disputes over the developmental

lineage of microglia (20, 21) [for translations of Hortega's work, see Ref. (22)]. In 1932, the first studies on microglia in human developmental tissue emerged (23–25). After Hortega and these initial studies, the investigation of microglial cells plunged into a long hiatus and received little attention from the neuroscience community, to be re-visited from the mid 1980s largely due to the technical advances of immunohistochemistry, with studies on the origin and identity of brain macrophages by Perry et al. (26) or Hickey and Kimura (27). Since then, the study of microglia has developed quickly and nowadays is one of the most active fields of neuroscience.

THE ORIGINS OF MICROGLIA

A critical phase in human embryonic development is gastrulation whereby the blastula becomes organized into a trilaminar structure that will yield out all the organs. The endoderm, forms the gut lining; the mesoderm generates the circulatory, cardiovascular and lymphatic systems; and the ectoderm generates both skin and the neuroectoderm, from which the nervous system develops (28, 29). Microglial origins have been a subject of debate and two dominant theories argued whether these cells originated from precursors in the neuroectoderm or the mesoderm. The neuroectodermal theory argues that microglia originate in the neurepithelium from pluripotent glioblasts and are, therefore, akin to astrocytes and oligodendrocytes in lineage (17, 30, 31). *In vitro* evidence of rodent neuroepithelial or astroglial cultures generating microglia supported this theory (32). Additionally, when bacteriophage lambda transgenic mice bone marrows (BMs) were transplanted into recipient murine neonates and adults, *in situ* hybridization and immunohistochemistry showed that the donors' BM-derived macrophages did not contribute to the adult microglial population arguing that microglia could be neuroectodermal in origin (33). The second theory argues that microglia originate in the yolk sac (YS) from hematopoietic stem cell progenitors akin to primitive macrophages and migrate to the brain rudiment as early as the fourth week of gestation in humans, the closure of the caudal neuropore occurring by 27 days post-conception [(17, 20), see also **Figure 1**]. Briefly, between the 15th and the 40th gestational days, the YS is the main hematopoietic organ and localized thickenings showing prospective blood islands are visible in the mesoderm by the 16th day and the peak of hematopoiesis is thought to be the middle of the fifth week of gestation (34–36). Rare histological reports demonstrate the occasional presence of macrophages prior to the onset of hematopoiesis in the yolk-sac by day 13, notably in the trophoblastic mesenchyme (37). By the sixth week of gestation, hepatic colonization by hematopoietic stem cells initiates subsequent hematopoiesis (38). Bone-marrow development does not occur until the 10th week of gestation, and it is well-established that it is not a hematopoietic organ until much later in term, and by the 50th post-conceptional day, YS hematopoiesis will have substantially decreased (34, 38, 39).

Evidence for the mesodermal theory comes from early studies showing the expression of macrophage markers in microglial cells such as FcγRI and CD45 suggesting an extra-cerebral origin and a shared ontology with macrophages within the mononuclear phagocytic system (49). In contrast, rodent studies have provided compelling evidence on the ontology of microglial cells. In mice, RUNX1$^+$ (Runt-related transcription factor 1 is used as a marker for precursor myeloid cells in the extra-embryonic YS) progenitors appear prior to embryonic day 8 in the YS and generate the entire microglial adult population independently of any bone-marrow derived contribution (50). These precursors are thought to be c-KIT$^+$ stem cells that eventually develop into mature CD45$^-$/c-KIT$^-$/CX3CR1$^+$ cells in a PU-1, IRF-8, and colony stimulating factor 1 (CSF-1R)-dependent manner before they acquire their identity as microglial cells upon invading the brain rudiment (50–52). In the human YS and mesenchyme sampled between 4 and 6 gestational weeks (gw), two populations of cells with different phenotypes have been distinguished: the majority carried both macrophage-associated (RFD7$^+$) and monocyte-associated markers (UCHM1$^+$) with no detectable HLA-DR antigen, and the minority expressed class II (HLA-DR and -DP) and no RFD markers, alluding to the possibility of distinct signatures in these populations prior to BM development (53). Overall, it is highly likely that human microglia derive from primitive YS macrophages similarly to rodents. This is an early process that precedes hepatic and BM hematopoiesis (54). Subsequently, mononuclear phagocytes issued from YS hematopoiesis colonize the various organs including the human brain, akin the model proposed for mouse microglial development (55).

MICROGLIAL POPULATION OF THE DEVELOPING BRAIN

The human developing forebrain, midbrain, and hindbrain become identifiable following the closure of the neuropore by 27 post-conceptional days (56–59) (see **Figure 1**). The first wave of entry of microglia into the developing brain predates the peak of neurogenesis and neuronal migration in the telencephalon (40, 60–66). Neural progenitors in the various regional germinal neuroepithelia along the ventricular zone (VZ) proliferate between 8 and 28 gw and migrate toward the cortical plate between 9 and 38 gw (40, 65). The pattern of migration predicts the identity of the neuron in the developing cortex. Generally, excitatory projection neurons originating in the VZ/subventricular zone (SVZ) migrate radially to the cortical plate (64, 67) while inhibitory GABA-ergic neurons originating in the VZ/SVZ and the ganglionic eminence migrate tangentially to the cortical plate before undergoing radial migration in the intermediate zone (IZ) though this does not appear to be universal as a subclass of interneurons migrates radially (68–71). Subsequent to assuming their cortical positions and acquiring their identity, synaptogenesis (26 gw onward) ensues to establish connectivity across the developing brain and this process carries on beyond birth (65, 72). Some, thought to be mesoderm-derived cells, make their route to the developing brain very early and three microglial entry routes have been described: the leptomeningeal route, the ventricular lumen route and the choroid plexus route. In the telencephalic wall, amoeboid microglial cells are detected in the ventricular lumen and VZ as early as gw 4.5. Concomitantly, amoeboid microglia also accumulate in the superficial marginal zone (MZ) presumed to enter from the pial surface and by 5.5 gw some amoeboid cells are also presumed to enter from the choroid

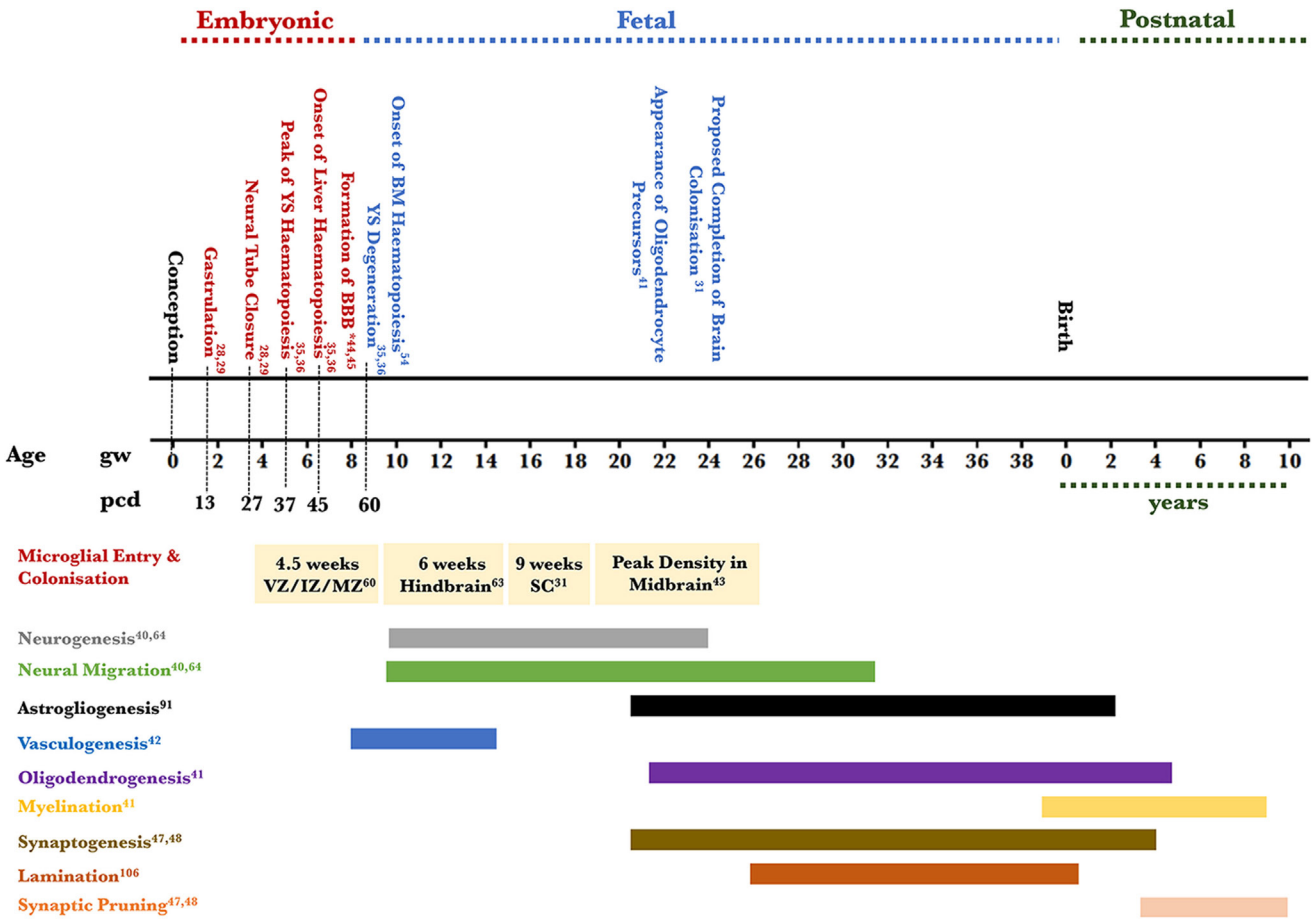

FIGURE 1 | Timeline of human microglial development. This figure places microglial entry into developing brain areas in the context of the approximate co-occurrence of cortical developmental events. Microglia are detected in the amoeboid state in the telencephalic ventricular, intermediate, and MZs and not the cortical plate at 4.5 weeks of gestation; microglia are detected in the ramified state in the hindbrain by 6 weeks; amoeboid microglia are detected in the SC by 9 weeks of gestation; the density of amoeboid microglia is highest in the mesencephalon (midbrain) by 22 weeks of gestation. The relevant references to each process are included (28, 29, 31, 35, 36, 40–45, 47, 48, 54, 60, 63, 64, 70, 91, 106). *Two barriers are included here: the BBB proper at the level of the endothelium of the cerebral blood vessels and the blood–CSF barrier at the level of the choroid plexus epithelial cells. The CSF–brain barrier is detected as early as 16 days through the appearance of strap junctions and is not shown on the timeline. Abbreviations: BBB, blood–brain barrier; BM, bone marrow; CSF, cerebrospinal fluid; IZ, intermediate zone; MZ, marginal zone; PCD, post conceptional days; GW, gestational week; SC, spinal cord; YS, yolk sac.

plexus into the parenchyma of the thalamic eminence. These cells are positive for ionized-calcium binding adaptor protein 1 (IBA-1) and are thought to be undifferentiated immature microglial cells (31, 60–63, 73). However, whether their transcriptional signature is already defined at that stage remains unresolved as some could belong to the macrophage lineage as there is some positivity for CD68, HLA-DR, and CD45. The density of microglial cells at the areas of entry during development does not correlate with their preponderance in areas of the adult brain. Therefore, it can be assumed that the cells would be migrating and proliferating or vice-versa (26). By the eighth gw and as the cortical plate thickens, intermediate rod-shaped microglial cells appear in the MZ and their density increases in the VZ, SVZ, IZ, and the subplate (74–77). Cells particularly in the IZ are suggested to be proliferating between 8 and 12 gw. By the 10th gw, and as fetal blood vessels appear in the telencephalic wall, some amoeboid microglia seem closely associated with the endothelium. Between the 9th and until the end of the 13th gw, proliferating amoeboid microglia aligned along the subplate-cortical plate boundary assume this location and do not enter the cortical plate well beyond its resolve by the third trimester (75, 78). These cells are suggested to support thalamocortical synaptic development (79). Between the 14th and 17th gw, already proliferated microglial cells cluster near or within the white matter at the junction between the thalamus and the internal capsule, the optic tract, and the junction between the internal capsule and the cerebral peduncle and later clusters are observed in the corpus callosum and the periventricular hypothalamus (60, 75, 80). By the 18th gw, intermediate microglia are suggested to migrate radially within the cortical plate to eventually develop into ramified gray matter microglia. Tangential migration occurs within the IZ yielding white matter microglia though interestingly, studies on quail retina suggest that initially, microglial cells

migrate tangentially along the various brain layers then assume a radial migration pattern to reach the deep brain parenchyma (81). Microglial cells with various morphologies (intermediate or ramified) populate the cortical mantle and are distributed differentially across the various layers (60, 82, 83). Finally, between the 19th and 30th gw, tangentially oriented microglial packed clusters with an intermediate rod-shaped morphology are reported in white matter tracts such as the corpus callosum and the external capsule. Their density decreases after the 35th gw (73). It is interesting to note that the onset of myelination coincides with the temporal window during which these cells increase in size and proliferate. In humans, the appearance of oligodendrocyte precursors dates to the 22nd gw with myelination commencing when these cells differentiate and become functional from the 28th gw (31, 41). It is, therefore, tempting to speculate that increased microglial presence during this time could be phagocytic during the onset of myelination particularly as some neurons will have migrated into the white matter and would, therefore, need to be cleared. The evidence for this comes from the activated shape of these microglia in these regions within 22–30 gw (84). Furthermore, various authors have also alluded to this temporal window as being when white matter is most susceptible to injury (60, 73, 85). Therefore, increased microglial presence could be seen as protective during this phase. Evidence for this hypothesis comes from pre-term fetal tissue assessed for white matter injury (periventricular leukomalacia) where abnormally high densities of activated microglia are seen in the same regions as the aforementioned clusters (73). By the end of gestation, ramified microglia can be seen across the cortical layers and the white matter shows a higher density of cells compared to the gray matter (86).

Very few studies have focused on characterizing microglial populations in the developing midbrain, cerebellum, and spinal cord (SC). In the cerebellum, its molecular and granular layers are apparent by the 12th gw (87). Ramified microglial cells have been detected as early as 7.5 gw (63) and as the telencephalic wall thickens, very few amoeboid microglial cells remain in the cerebellum (88). It has been suggested that the caudorostral appearance of the vasculature (cerebellum to telencephalon) coincides with increases in microglial densities (12, 42, 63). Much less is known about the mesencephalon (future midbrain) though a couple of studies have highlighted that microglial ramified densities reach a record high during development in this area in comparison with other areas (12, 43, 89). Finally, in the SC, microglia are suggested to colonize the gray and white matter by the ninth gw (31). Their pattern of migration into the cord is thought to follow an outside-in pattern (from the meninges into the gray/white matters) (31). Toward 18–24 gw, SC gray matter microglia are ramified while white matter microglia are amoeboid (90).

Overall, microglial cells enter the developing brain very early preceding neurogenesis, neuronal migration, and gliogenesis in the regional neuroepithelia (40, 91). As development is a highly dynamic and metabolically demanding period, it is likely that apoptosis is coupled with proliferation as has been documented in the cortical subplate for example at the end of the first trimester (92). It is also interesting to consider the human blood–brain barrier (BBB) across cerebral endothelial cells and the CSF brain barrier across the neuroependyma, which shows mature tight junctions as early as the seventh gw as demonstrated by freeze-fracture studies on human embryos of that age (44, 45, 93, 94). Additionally, Gröntoft (and later confirmed by experiments on rabbit fetuses) clearly demonstrated that trypan blue in a 9 gw fetus did not reach the brain if injected in less than 10 min from fetal placental separation (cesarean section delivery) indicating an intact BBB (95). Thus, this may suggest that microglial populations could be expanding as a population from residing cells beyond the closure of the BBB. Furthermore, knowing that some areas of the brain develop much later than others (the cerebellar primary fissure is only visible from the 12th gw, for example) and after BBB closure has been completed, investigating microglial dynamics in this area may shed light on why the adult human cerebellum may contain much less microglial cells in comparison with gray and white matter regions.

FUNCTIONAL ROLES OF MICROGLIA DURING BRAIN DEVELOPMENT

The early colonization of the developing brain by microglia antecedes neurogenesis, neuronal migration, and many other cellular processes (see **Figure 1**) suggesting that these cells are very likely to play pivotal roles in mediating the normal occurrence of some of these events. Multiple lines of functional evidence, predominantly from rodent studies, support this. First, maternal administration of liposomal clodronate to deplete fetal microglia or a maternal immune challenge to activate microglia affect the size of the neuronal precursor pool in the SVZ during the late stages of cortical neurogenesis (96). This has been demonstrated in the rat fetus and highlights the importance of the phagocytic role of microglia in restricting the number of neuronal precursors during development. Furthermore, microglia are thought to promote the apoptosis of Purkinje cells (through superoxide anion signaling during late cerebellar development) in the mouse (97), granular cells in the rat hippocampus (98), and ganglionic cells in the avian retina (81). Second, the transient blockade of CSF-1R at the onset of yolk-sac microglial progenitor production (E6.5) in the mouse embryo alters the normal positioning of a subclass of cortical interneurons (Lhx6) and causes aberrant dopaminergic axonal outgrowth (99). This supports the role of microglial cells in the migration of some cortical interneurons to their correct laminar position and provides novel evidence of the role of these cells in axonal outgrowth. Third, the embryonic absence of microglia in PU.1 knock-out mice is associated with a decreased rate of cortical astrocyte differentiation, suggesting an association between these two cell types (46). In fact, a close association between GFAP-vimentin positive cells and microglia has been already documented in the ninth gw human SC though the functional implication of this has not been elucidated (31). Fourth, challenging the immune system of pregnant dams (by lipopolysaccharide injection at E15.5) or mutating part of the microglial TREM2 signaling pathway results in defasciculation of the corpus callosum in mouse embryos (100). Furthermore, microglia are associated during development with axonal tracts, and this has been repeatedly shown in gestational human tissue (31, 60, 73). Hence, these findings suggest a role of microglia in axonal pruning and the

support of myelination. Finally, fractalkine receptor (CX3CR1) knock-out mice have delayed synaptic pruning demonstrated by a transient excess of dendritic spines concomitant with decreased microglial numbers during development. Functionally, these mice had also reduced synaptic efficiencies in hippocampal long-term potentiation (101). Thus, microglial cells have also a pivotal role during development in synaptic refinement.

Altogether, microglial cells participate actively in various processes during brain development. Functional human studies are very difficult to conduct. However, and as previously discussed, histopathological studies on human embryonic and fetal tissues concur about the distribution of microglial cells in association with some key developmental processes including neuronal migration, white matter tract formation, vasculogenesis, as well as astrocyte formation and synaptic pruning.

LIMITATIONS OF EXISTING METHODS AND FUTURE PROSPECTS

Postmortem studies on human tissue are invaluable for investigating the antigenic features and morphology of microglial cells across various developmental ages. The identified studies in this review give us a qualitative insight into microglial dynamics during development. However, there are multiple caveats. First, though the total number of cases investigated in each study varied between $n = 4$ and $n = 47$ cases, it is extremely difficult to interpret the results when a single case is used to generalize over one gestational age. This is mainly because tissue is scarce. Additionally, and in cases of spontaneous abortion, the lack of neuropathological abnormalities may not exclude the fact that pregnancy loss could be the outcome of an underlying condition and that could also bias microglial findings linked to death. To date, most neuropathological studies have focused on predominantly qualitative methods to assess microglial populations in human embryonic/fetal tissues and with immunological (CD45, MHCII, CD68, IBA-1) or histochemical markers (RCA-1) that do not offer the possibility to distinguish between microglial cells and macrophages. This is likely to be a challenge for the field as development is highly dynamic, and we do not have specific markers to distinguish between cell lineages. New methods are needed to help identify a possible developmental microglial signature in the like of RNA-sequencing and single cell sorting methods from embryonic/fetal human tissue. To the author's knowledge, only one study has emerged on this topic (102). At present, these tools are being used to identify enriched genes in human adult microglia against perivascular macrophages, for example, and to distinguish human microglial cells from rodent microglia (9, 10). Quantitative methods to assess microglial numbers and morphology in sections are needed to calculate the degree of proliferation, apoptosis, and to establish the microglial/macrophage marker developmental profile. These are much more likely to support our understanding of the regional heterogeneity of microglial cells during development and the dynamics of this population. Furthermore, the relevant mathematical models applied to these data would allow for covariate correction such as the post-mortem interval, brain weight, gestational age, gender, and anatomical location in order to reveal main effects on microglial numbers as well interactions between these independent variables. Significant advances in the field would be made with tightly controlled tissue samples from elective terminations and well-powered sample sizes in addition to emphasis on the previously mentioned points.

Finally, microglia feature as part of the pathophysiological signature of various neurodevelopmental disorders such as schizophrenia (5), intellectual disability (103), autism (104), and Rett's syndrome (105). Therefore, understanding their precise dynamics and how they contribute to altering brain networks is likely to help us design therapeutic strategies to enhance their activity, limit the damage that could be caused by their activation, or use them as diagnostic biomarkers in disease.

CONCLUSION

In the past decade, the field of study of microglia has rapidly expanded, and although rodent studies provide the opportunity to investigate how these cells populate the brain, develop, and proliferate, studies on the developing human brain are needed, particularly as the human and rodent adult brains have different microglial signatures and regional distributions. It remains unclear how these differences contribute to function and further studies correlating anatomy with function are needed.

AUTHOR CONTRIBUTIONS

DAM and DG-N conceived the structure and content of the manuscript. DAM wrote the manuscript and prepared the figures. DAM and DG-N drafted the manuscript.

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MINI REVIEW
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A Brief History of Microglial Ultrastructure: Distinctive Features, Phenotypes, and Functions Discovered Over the Past 60 Years by Electron Microscopy

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The first electron microscope was constructed in 1931. Several decades later, techniques were developed to allow the first ultrastructural analysis of microglia by transmission electron microscopy (EM). In the 50 years that followed, important roles of microglia have been identified, specifically due to the ultrastructural resolution currently available only with EM. In particular, the addition of electron-dense staining using immunohistochemical EM methods has allowed the identification of microglial cell bodies, as well as processes, which are difficult to recognize in EM, and to uncover their complex interactions with neurons and synapses. The ability to recognize neuronal, astrocytic, and oligodendrocytic compartments in the neuropil without any staining is another invaluable advantage of EM over light microscopy for studying intimate cell–cell contacts. The technique has been essential in defining microglial interactions with neurons and synapses, thus providing, among other discoveries, important insights into their roles in synaptic stripping and pruning *via* phagocytosis of extraneous synapses. Recent technological advances in EM including serial block-face imaging and focused-ion beam scanning EM have also facilitated automated acquisition of large tissue volumes required to reconstruct neuronal circuits in 3D at nanometer-resolution. These cutting-edge techniques which are now becoming increasingly available will further revolutionize the study of microglia across stages of the lifespan, brain regions, and contexts of health and disease. In this mini-review, we will focus on defining the distinctive ultrastructural features of microglia and the unique insights into their function that were provided by EM.

Keywords: microglia, ultrastructure, electron microscopy, correlative light and electron microscopy, 3D ultrastructure

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INTRODUCTION

Microglia are the only immune cells that permanently reside in the brain. Originally believed to mediate inflammatory responses to infection (1), trauma (2), ischemia (3), or neurodegenerative disease (4), recent studies identified microglia as crucial actors in the proper development and maintenance of neuronal circuits (5). del Río-Hortega provided the original morphological description

Abbreviations: 2p, two-photon; EM, electron microscopy; TEM, transmission electron microscope; SEM, scanning electron microscope; DAB, diaminobenzidine; Iba1, ionized calcium-binding adapter molecule 1; CLEM, correlative light and electron microscopy; AD, Alzheimer's disease; SBEM, serial block-face scanning electron microscope; FIB-SEM, focused-ion beam coupled with scanning electron microscope.

of microglia at the turn of the twentieth century, having modified Golgi's silver stain to identify microglia (6). His manuscripts have recently been translated into English and annotated (7). Early research into microglial physiology prompted researchers to posit hypotheses that still hold true: microglia are phagocytic; they are capable of generating inflammation in response to infection; they may be responsible for some aspects of neurodegenerative disease; they originate outside of the brain and colonize it early in development (8). Between the early twentieth and twenty-first centuries microglia remained mainly uninvestigated as a cell type [reviewed in Ref. (9)], until Davalos et al. and Nimmerjahn et al. uncovered their incredibly dynamic processes in the adult brain under physiological conditions using two-photon (2p) microscopy (10, 11). Following this discovery and with the development of genetic tools to specifically identify microglia and their progeny (12–14), high throughput gene-expression analysis (15–18), and investigation into expression of cell surface receptors (19, 20), researchers have completed a whirlwind of studies in an attempt to unravel microglial roles in a myriad of healthy and disease processes (21). Recent developments in super-resolution and 2p microscopy have provided insight into microglial interaction with dendritic spines (22–24). However, genetic manipulations required for marker expression in neurons and microglia can induce cellular stress and impair normal functions (25–27). Electron microscopy (EM) can be used to investigate the unique ultrastructure of microglia and their relationship with synapses, and identify their phagocytic cargo without any immunohistochemical or genetic labeling. While super-resolution microscopy has surpassed the diffraction limit of light microscopy, its resolution is still insufficient to discern samples smaller than 50 nm, especially in the *z*-dimension, and requires specific labeling probes to prevent steric hindrance from influencing the resulting image (28). In this review, we will focus on the use of EM to unravel structural and functional mysteries of microglia and their interaction with healthy and diseased brain tissue.

HISTORY AND DEVELOPMENT OF EM

Electron microscopy utilizes focused electron beams to illuminate the subject of interest. Since an electron's wavelength is up to 100,000 times shorter than a photon's, EM is capable of resolving atomic structures, while most light microscopes are diffraction-limited to 500 nm resolution.

Hans Busch, a pioneer in the field of electron optics, laid the theoretical groundwork for EM by determining the motion of electrons in a magnetic field and the potential to focus electron beams (29). The first EM was invented by Knoll and Ruska in 1932, based on the Bush's published theories (30). The first transmission electron microscope (TEM) functioned by projecting electrons through a thin sample and onto film, and investigating the regions of the sample that were electron-permissive versus electron-dense. Shortly after TEMs were developed, the first scanning electron microscope (SEM) was invented in 1940 (31). SEM differs from TEM as it visualizes electrons that are scattered off the surface of the specimen instead of electrons that pass through the specimen.

Over the following three decades, scientists perfected multiple ways to process and preserve biological samples in order to garner useful images of *in situ* tissue preparations (**Figure 1**). Aldehyde fixation cross-links proteins in tissues (32, 33), while osmium tetroxide fixation mainly preserves lipids and renders membranes electron-dense (34). The development of transcardiac perfusions provided fast delivery of fixatives to deep regions of the brain and

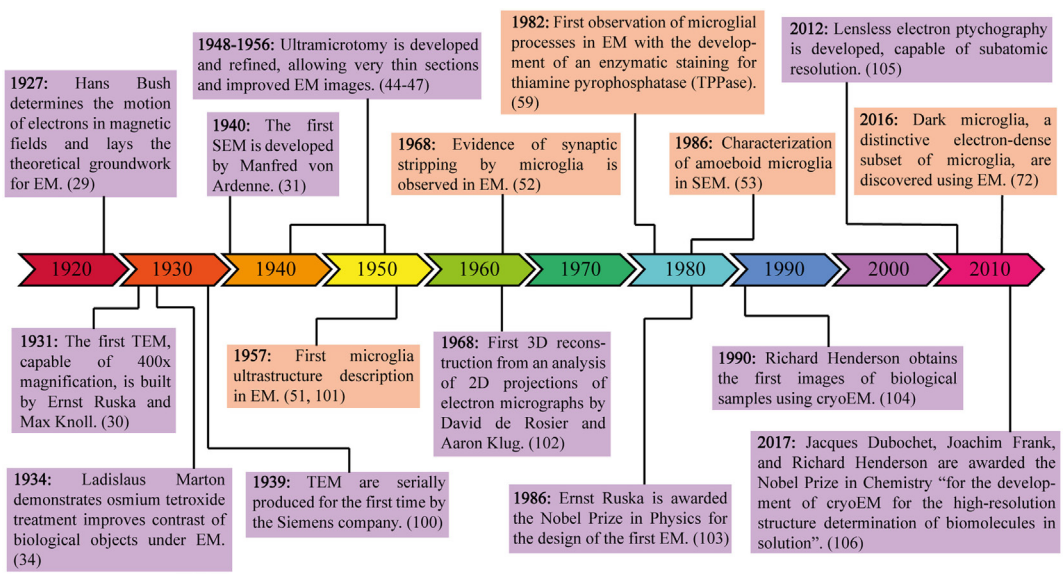

FIGURE 1 | Milestones in electron microscopy (EM) engineering and discovery. This timeline highlights the major theoretical and experimental advances in EM, from the invention of the first electron microscope to the 2017 Nobel Prize in Chemistry for the discoveries leading to cryoEM. Purple frames contain information about the development of technology required for EM, while orange frames contain information about microglial discoveries made possible through the use of EM.

other biological tissues, arresting any possible degradation that may have occurred in diffusion-dependent fixation techniques (35–37). However, using aldehydes or other fixatives results in tissue shrinkage and loss of extracellular space. This can be avoided by freeze substitution, a type of cryoEM: flash-freezing the tissue of interest followed by fixation performed at very low temperatures (38). Fixing the specimen in buffers that match the osmolarity of the tissue of interest can preserve extracellular space (39). Alternatively, if the specimen and chamber of the EM are kept below −140°C, samples can be visualized without any fixation (40, 41). Cell viability assays and staining for cell surface markers can be performed on live cells in suspension prior to deposition onto TEM grids and flash-freezing (42, 43).

Particularly important for TEM imaging was the development of ultramicrotomy, which allowed ultrathin (50–80 nm) sections to be cut from larger specimens, thus improving resolution and focus (44–47). These ultrathin sections allowed researchers to visualize ultrastructural images of various biological samples by capturing the transmitted electrons after they passed through the specimen onto films. The conventional protocol to prepare biological tissue for TEM is well explained by several groups (48–50).

EM AND MICROGLIA

In 1957, the first ultrastructural image of microglia in the rat parietal cortex was published (51), and in 1968, TEM images showed microglia physically separating presynaptic terminals from postsynaptic dendrites or neuronal cell bodies, a term defined as synaptic stripping (52). The first TEM images of microglia uncovered clues to the dynamic nature of these cells, decades before 2p microscopy discovered their movements to survey the brain parenchyma in real-time. Cultured microglia investigated using SEM identified many tiny processes projecting directly from cell somas, and draw stark attention to the two-dimensional stressors placed on cells in culture (53). Pioneering studies in EM identified many unique characteristics of microglial cell bodies, before any cell-specific immunological studies were developed.

Microglial cell bodies can be discerned from those of other cell types by their small size (3–6 μm), electron-dense cytoplasm, and characteristically bean-shaped nuclei. They also display a distinct heterochromatin pattern. A thick, dark band of electron-dense heterochromatin is located near the nuclear envelope, with pockets of compact heterochromatin nets throughout the nucleus. These nets are often visualized as small islands of dark heterochromatin within a sea of more loosely packed, lighter euchromatin within the central part of the nucleus (54, 55). Microglial cell bodies have a very thin ring of cytoplasm separating their nuclei from their cell membranes, and contain few organelles within a single ultrathin section, but those visible are mostly mitochondria, long stretches of endoplasmic reticulum, Golgi saccules, and lysosomes (54, 56, 57). They are often phagocytic and contain lipidic inclusions, especially in older animals (58) (**Figures 2A,D**).

The development of microglial-specific stains compatible with EM has been a major aid in determining their functions *in situ*. Labeling microglial membranes and cytoplasm, originally with enzymatic reactions and more recently with immunoEM, allowed researchers to investigate microglial processes in animal models and human postmortem tissue (57, 59, 60). Current immunoEM studies utilize either diaminobenzidine or gold-conjugated antibodies (or colabeling using both) to deposit electron-dense precipitate and identify proteins of interest (22, 61). Ionized calcium-binding adapter molecule 1 (Iba1) is often used to identify microglia/macrophages within the brain (62). After much study using immunoEM to identify their main characteristics (22), trained researchers can identify microglial processes based solely on their unique ultrastructure. Microglia's ramified projections are long, thin, and almost never contiguous with their cell bodies in ultrathin sections examined by TEM (**Figures 2B,C**). They are often in close, direct contact with neuronal cell bodies, or separated only by a very thin astrocytic process (22, 57, 59, 63). A single microglial process can contact multiple synaptic elements, and interacts with axon terminals, dendritic spines, perisynaptic astrocytic processes, and encircles parts of synaptic clefts (22, 63). Their processes often perform extracellular degradation, visible as pockets of extracellular space sometimes containing pinpoints of membrane degradation. They frequently contain vacuoles or multivesicular bodies, long stretches of endoplasmic reticulum, and phagocytic inclusions (**Figure 2C**).

Microglia promote proper neuronal wiring and activity, and EM studies were vital for discovering their role in development and maintenance of functional neuronal connections (21). Elegant EM studies demonstrated that glia (performing the functions of microglia) in *Drosophila* (64), macrophages and microglia in zebrafish (65), as well as microglia in rodents (66–68) phagocytose degenerating axonal tracts, axon terminal fragments, and dendritic spines during development of the thalamus, cerebral cortex, and hippocampus. Interestingly, no phagocytic interactions between microglia and synapses were identified in TEM studies of a mouse model of prion disease (69), although immunoEM was not performed and microglial processes may have been overlooked. Microglia also phagocytose putative neuronal debris following saponin-induced cholinergic cell death in rats (70). Sequential EM images are required to verify phagocytic cargo is fully enclosed within a microglial process and has been demonstrated for phagocytosis of synaptic elements by both microglia and astrocytes (22, 67, 71). Automation of sequential EM using knives or focused-ion beams inside SEM chambers can provide nanometer-scale resolution images of microglia in 3D.

Recent TEM studies have uncovered a new microglial phenotype, named dark microglia. These dark microglia share many ultrastructural characteristics (including cell size, immunohistochemical markers, and phagocytic phenotype) with healthy microglia, yet appear strikingly different under TEM. Their cell bodies can be quickly identified by their condensed, electron-dense cytoplasm that makes them appear as dark as mitochondria. Dark microglia display many signs of cellular stress, including nuclear and chromatin condensation and dilation of their endoplasmic reticulum (**Figure 2F**). Additionally, they are present in greater numbers in pathological contexts often associated with neuronal dystrophy and distress. They have been identified in the APP-PS1 mouse model of Alzheimer's disease (AD), aged mice, animals subjected to social defeat stress, fractalkine receptor-deficient mice, and mouse models of schizophrenia (72, 73). They show reduced expression levels of some microglial markers, including

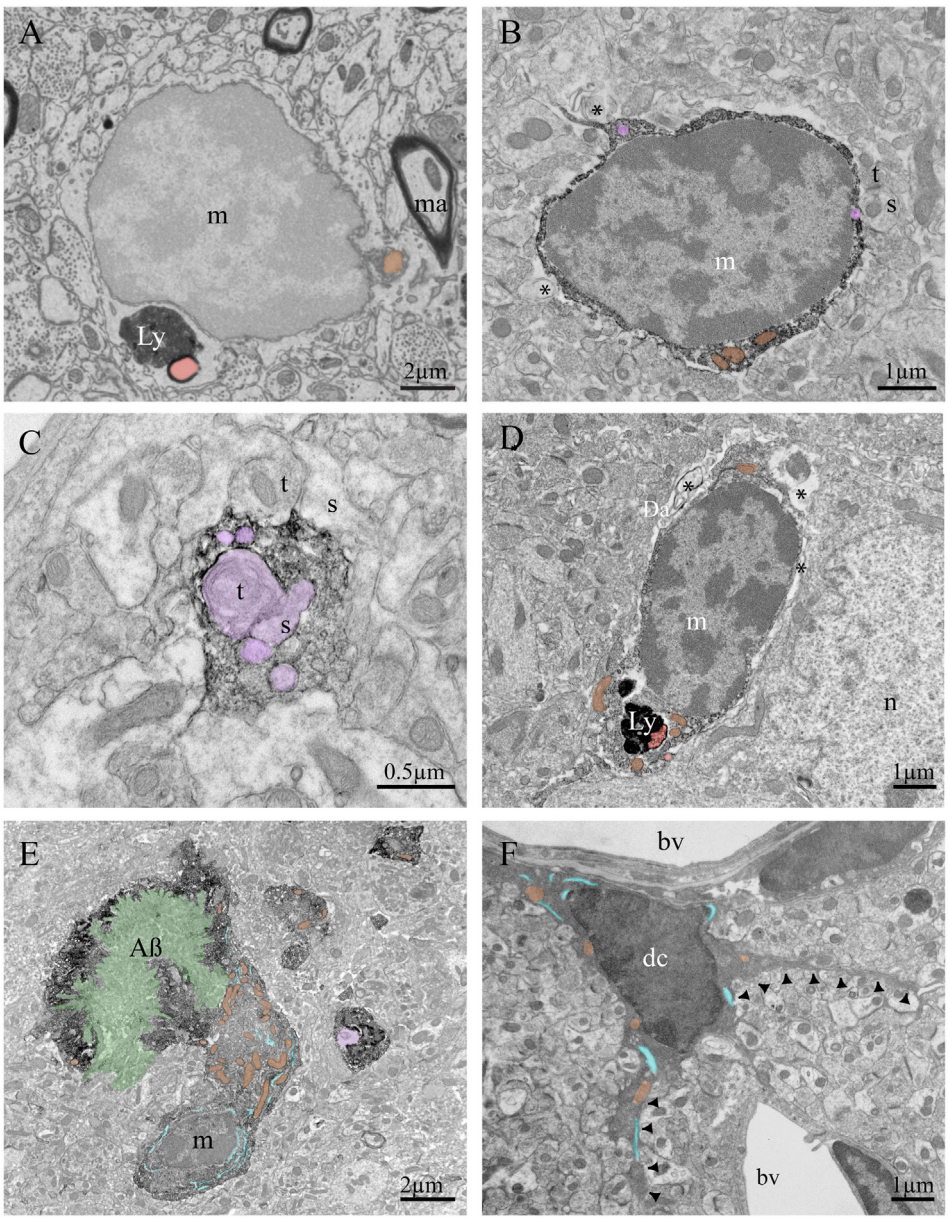

FIGURE 2 | Ultrastructural features of murine brain microglia in health and disease. Example of microglia imaged using a focused-ion beam coupled with scanning electron microscope without any immunostaining **(A)**, containing lipofuscin granules (Ly) and a lipid body (Lb). Diaminobenzidine staining against ionized calcium-binding adapter molecule 1 (Iba1) creates a dark immunoprecipitate in the cytoplasm as shown by transmission electron microscopy (TEM) **(B–E)**. Iba1 staining allows identification of microglial processes in fractalkine receptor-knockout mice, for instance, allowing researchers to investigate their contacts with synaptic terminals and study phagocytic inclusions. **(B)** A microglial cell body in an APP-PS1 mouse is contacting a synapse between two axon terminals and a dendritic spine, as well as juxtaposing cellular debris. **(C)** A microglial process in a C57Bl/6 mouse contains several inclusions, notably an axon terminal making a synaptic contact on a dendritic spine. **(D)** A microglial cell body in a mouse model of Werner syndrome juxtaposes myelin debris and contains lipofuscin granules. **(E)** A microglial cell body in an APP-PS1 mouse is found in intimate contact with an amyloid beta plaque. **(F)** Example of dark microglia observed by TEM in a stressed fractalkine receptor-deficient mouse, characterized by its dark cytoplasm and thin processes projecting from the cell body (black arrowheads). Symbols and abbreviations: m, microglia; n, neuron; dc, dark microglia; t, axon terminal; s, dendritic spine; bv, blood vessel; Ly, lipofuscin; Da, degenerated axon; ma, myelinated axon; AB, amyloid-beta plaque. Asterisk (*) denotes evidence of cellular debris undergoing digestion in the extracellular space. Pseudocolor code: phagocytic inclusions = purple, examples of dilated endoplasmic reticulum = blue, examples of mitochondria = orange, amyloid-beta plaque = green, lipid bodies = red.

Iba1, but are strongly immunopositive for others, including complement receptor subunit CD11b and microglia-specific antibody 4D4. Most dark microglia located near amyloid plaques in APP-PS1 mouse model express TREM2, though dark microglia in other disease models do not. While normal microglia rarely have contiguous processes attached to their somas in ultrathin sections, dark microglia show many long, thin processes encircling dystrophic neurons, wrapping around synaptic structures,

investing themselves deep into amyloid beta plaques, and interacting with synapses in regions of high synaptic turnover (72). Although they display many signs of cellular stress, they have never been found expressing apoptotic or necrotic cell markers. Dark microglia are often located near blood vessels, which could imply a possible peripheral origin or perhaps a route of egress for the stressed cell to leave the brain parenchyma (**Figure 2F**). As there is not yet a definitive marker of dark microglia, they can only be investigated with EM, highlighting its relevance in modern microglial biology studies.

CORRELATIVE LIGHT AND ELECTRON MICROSCOPY (CLEM)

The combination of both light microscopy and EM can be used to uncover more information than either technique individually. CLEM was first used in 1969. Silver staining originally described by Río-Hortega was used to identify and investigate microglia in light microscopy. After confirming microglial-specific staining, researchers investigated ultrathin sections under TEM and published the first description of microglial ultrastructure (54).

Electron microscopy is currently used to unravel details and variations in ultrastructure that cannot be investigated with light microscopy (**Table 1**). Light microscopy is often used to detect changes in microglial density and morphology in health and disease (74), and to identify particular regions of interest. After identifying a region of interest, such as one affected by hypoxia in stroke or amyloid-beta positive plaque-containing tissue in AD, EM can delve further into specific changes in microglial ultrastructure, cellular viability and stress, all without requiring further immunostaining markers (75, 76). EM can also reveal structures which are not otherwise visible, and discern subcellular localization of proteins and mRNA using immunostaining, *in situ* hybridization, or *in situ* RT-PCR (57, 77, 78). EM was recently used to clarify microglial process fragmentation observed with light microscopy in postmortem human tissue from an individual suffering from AD. Unexpectedly, EM studies revealed no fragmentation as the two parts of the microglial process were linked by a cytoplasmic bridge, thus invalidating the original hypothesis (79).

Light microscopy can also be performed on living cells prior to investigation with EM to tie temporal information to ultrastructural resolution. The technique used is a specific type of CLEM. Live imaging using 2p microscopy studies cellular relationships, interactions with the surrounding environment, and intracellular dynamics in real-time; but lacks complex ultrastructural information. These imaging techniques are also limited to genetically encoded or virally introduced cell-specific fluorescent markers, which may introduce phenotypic changes on their own (26). EM can uncover structural information, but the specimen must be fixed (or flash-frozen for cryoEM), and can only be investigated as a snapshot moment in time. CLEM integrates imaging of fluorescent proteins in live cells with the ultrastructural resolution of EM. After live-cell imaging is performed, various fixation and staining techniques can be employed to investigate ultrastructure in the same tissues. van Rijnsoever and colleagues used CLEM to study the endolysosomal system by confocal microscopy followed by cryoEM to image protein structures with nanometer resolution (85). This technique could be used to obtain insight into microglial proteins, phagocytic machinery, and organelle biogenesis.

It is also possible to combine EM with 2p microscopy. While 2p studies allow investigation of live microglial interactions with nearby neurons, EM performed afterward can study the intimate contacts between microglia and synaptic elements, and their changes in response to various behavioral experiences and pathologies (22, 63, 86–88). Light microscopy also informs EM studies, making it much easier to solve needle-in-haystack problems and identify rare events within the neuropil. For example, an Alzheimer's study injected animals with methoxy-X04, a blood–brain barrier permeable amyloid-beta fluorescent marker. Researchers then selected sections containing the region of interest known to contain amyloid-beta, thus increasing the likelihood of finding plaque-associated microglia in ultrathin sections (**Figure 2E**) (50). Similarly, fluorescent microscopy can be used to target a specific microglial population to be analyzed in EM. Bechmann and Nitsch fluorescently labeled axons prior to performing entorhinal lesions and traced the clearance of degenerating tissue by identifying the fluorescent compound within nearby microglia. By focusing EM studies on regions containing fluorescently labeled microglia, they were able to investigate the subpopulation of microglial cells which had phagocytosed degenerating axons (89).

Another CLEM technique is the use of light microscopy in correlation with cryoEM. A study utilizing both techniques recently discovered the native folding of herpes simplex virus as it moved throughout axons. 3D visualization permits analysis of vesicle fusion and actin bond formation (90). CryoEM was also recently used to image Golgi apparati in two different conformations within neurons (91) and investigate minor changes in ultrastructure following intracerebral injections (a common technique used to introduce vectors into mouse models) (92). Cryo-fixation preserves extracellular space, especially notable at synapses and blood vessels (93). This method could be used to determine native folding and unfolding of proteins within microglia, to better understand their morphological and functional changes in various disease conditions.

THE FUTURE OF EM AND MICROGLIA

The past 15 years have seen a whirlwind in EM development. Previously, when investigating 3D ultrastructure, serial ultrathin sections were manually cut and collected at the ultramicrotome, imaged individually onto film under a TEM, and painstakingly reoriented and collated prior to analysis (94). As digital imaging improved, TEMs were outfitted with digital cameras allowing for faster imaging, but the electron beam of the TEM could still deform ultrathin sections, making perfect alignment of sequential sections almost impossible. Developments in SEM opened the door for array tomography studies on ribbons of serial ultrathin sections, allowing CLEM on the same tissue, and solving the problem of deformation introduced when using TEM (81).

The first major revolution in 3D ultrastructure imaging came when Denk and Horstmann engineered a working diamond knife

TABLE 1 | Types of EM.

Type of EM	Typical sample preparation	Maximal resolution	Advantages	Disadvantages
Transmission electron microscopy (TEM) (49)	– Fixation with aldehydes and plastic resin embedding – Manually cut ultramicrotomy (thin sections of 50–80 nm stored on metal grids)	Nanometer resolution in *x*, *y* Resolution in *z* limited by section thickness	– Tissue can be archived and reimaged – Block of tissue may be saved and recut – Highest resolution and magnification – Osmium fixation is not required	– Biological specimens must be fixed with gluteraldehyde or acrolein – Low throughput – Electron beam can cause deformation of ultrathin tissue sections – Smaller magnification range (680× to greater than 30,000×)
Scanning transmission electron microscopy (STEM) (49)	– Fixation with aldehydes, strong post-fixation with osmium (OTO), and plastic resin embedding – Manually cut ultramicrotomy (thin sections of 50–80 nm stored on metal grids)	Nanometer resolution in *x*, *y* Resolution in *z* limited by section thickness	– Tissue can be archived and reimaged – Block of tissue may be saved and recut – Faster imaging throughput than traditional TEM – Large magnification range (20× to greater than 30,000×)	– Biological specimens must be fixed with gluteraldehyde or acrolein – Stronger osmium fixation required than traditional TEM – Electron beam can cause deformation of ultrathin tissue sections – Risk of tissue destruction is higher than with traditional TEM
Scanning electron microscopy (SEM) (80)	– Dehydration – Strong post-fixation with osmium (OTO) if material contrast imaging is desired – Entire specimen (entire insect, dissected organ, etc.) mounted on a stub of metal with adhesive – Coated with a conductive metal	Nanometer resolution in *x*, *y*, and *z* for surface topography	– Tissue can be archived and reimaged – Large magnification range (20× to greater than 30,000×) – Can create images of up to several cm^3, which provides a good representation of the 3D shape of the specimen – Secondary electron detector measures surface topography – Backscatter electron detector measures material contrast (i.e., cell membrane versus cytoplasm)	– Biological specimens must be fixed with gluteraldehyde or acrolein – Image is created using scattered electrons and limited to the surface of the specimen
Scanning electron microscopy with array tomography (81)	– Fixation with aldehydes, strong post-fixation with osmium (OTO), and plastic resin embedding – Manually or automatically cut serial sections ultramicrotomy (thin sections of 50–80 nm stored on silicon chips or magnetic tape)	Nanometer resolution in *x*, *y* Resolution in *z* limited by section thickness	– Tissue can be archived and reimaged – Image large and serial sections – Large magnification range (20× to greater than 30,000×) – Compatible with correlative light-EM imaging – No deformation of tissue, making serial reconstruction simpler	– Serial section cutting and collecting is technically challenging – Stronger fixation required for proper material contrast
Focused-ion beam–scanning electron microscopy (FIB–SEM) (82)	– Fixation with aldehydes, strong post-fixation with osmium (OTO), and plastic resin embedding – Prepared tissue specimen (3–10 mm^2 wide × 3–10 mm^2 tall × 50–75 µm thick) mounted on a stub of metal with adhesive – Coated with a conductive metal	Nanometer resolution in *x*, *y* Up to 5 nm resolution in *z* (83)	– Nanometer resolution (less than 5 nm per pixel) in all three dimensions – Simplest serial image reconstruction	– The entire tissue block must be mounted and cannot be resectioned – Limited to a very small area, usually less than 15 µm × 15 µm – Smaller magnification range (400× to greater than 30,000×) – The sample is destroyed as it is imaged and cannot be reimaged
CryoTEM (84) CryoSEM (84)	– High-pressure freezing – Manually or automatically cut sections using cryo-ultramicrotomy (40–100 nm thick)	Nanometer resolution in *x*, *y* Resolution in *z* limited to section thickness	– No fixation required – Allows imaging of specimens in a native-like state	– Technically challenging – The sample must be flash-frozen to preserve native protein folding – The sample must remain frozen through entire process

Table of the major types of electron microscopy (EM) described in this mini-review, highlighting sample preparation, maximal resolution, magnification power, and advantages and disadvantages to each technique. Typical sample preparation is provided for each method, but fixation with aldehydes can be avoided if the researchers instead flash-freeze samples and perform freeze-substitution following sample collection.

into the chamber of a SEM to perform serial block-face scanning electron microscope (SBEM) (95). With this approach, the block face is imaged, a 50-nm section is cut away, followed by another image. Peddie and Collinson recently reviewed the many types of 3D EM and its applications to biological tissues (96). A decade after SBEM was invented, it allowed researchers to confirm microglial synaptic stripping (97). Research in a mouse model of multiple sclerosis used SBEM to unravel different roles of microglia *versus* infiltrating monocytes very early in the disease. The authors performed SBEM and differentiated resident microglia from invading myeloid cells by their ultrastructural differences (changes in mitochondrial makeup, nuclear shape, and presence of osmiophilic granules) in order to determine that demyelination in experimental autoimmune encephalitis is initially performed by invading monocytes, while resident microglia did not contribute to the early stages of inflammation (98). More recently, focused-ion beam coupled with SEM (FIB-SEM) has improved resolution from 50 nm to less than 10 nm in the *z*-dimension (99). By employing a focused-ion beam to atomize a very thin layer from a small (usually less than 500 μm^2) area, researchers may image at 5 nm resolution in *x*, *y*, and *z* (82).

Both SBEM and FIB-SEM are capable of investigating neuronal ultrastructure, and can follow a single process through several microns of neuropil, but FIB-SEM is also capable of resolving synaptic vesicles, lysosomes, and phagosomes in three dimensions. If the FIB-SEM process began within a microglial cell body, researchers could trace fine microglial processes through several microns of neuropil, without having to perform immunoEM. This offers a better chance to investigate lipidic inclusions and other pathological changes in organelles obscured by electron-dense precipitates used in immunoEM.

In addition to technological advances in both SEM and TEM, the rapid development of cryoEM techniques described here could uncover native protein structures within microglia. It could additionally pave the way for discoveries into the snapshot of microglial–neuron and microglia–glia interactions without requiring fixatives, and without the corresponding tissue deformation that occurs with rapid fixation currently required to preserve ultrastructure. While fixatives and ultrathin sections required for EM are not compatible with post-imaging analysis of RNA or proteins, future iterations of CLEM (perhaps cryoCLEM) and advances in single-cell mRNA isolation may be able to isolate subcellular tissue fractions for further analysis. Armed with these new tools, biologists may investigate the complex interactions between glia and neurons in a number of diseases. The unique nature of EM allows researchers to characterize unique ultrastructural characteristics of microglia and other immune cells, and uncover possible paths for therapeutic intervention.

AUTHOR CONTRIBUTIONS

JS and MET conceived the ideas and drafted the manuscript to which all authors contributed. KP and FG-I created the figures. All authors read and approved the final version of the manuscript.

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Deconvolution of Human Brain Cell Type Transcriptomes Unraveled Microglia-Specific Potential Biomarkers

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Microglial cells form a context-dependent network of brain immunoeffector cells. Despite their indispensable roles, unresolved questions exist around biomarker discovery relevant to their cellular localization, self-renewing potential, and brain developmental dynamics. To resolve the existent gap in the annotation of candidate biomarkers, we conducted a meta-analysis of brain cells using available high-throughput data sets for deciphering microglia-specific expression profiles. We have identified 3,290 significant genes specific to microglia and further selected the top 20 dysregulated genes on the basis of *p*-value and $\log_2$FC. To this list, we added 7 known microglia-specific markers making the candidate list comprising 27 genes for further downstream analyses. Next, we established a connectome of these potential markers with their putative protein partners, which demonstrated strong associations of upregulated genes like Dedicator of cytokinesis 2 (*DOCK2*) with early/mature microglial markers such as Sphingosine kinase 1 (*SPHK1*), *CD68*, and *CD45*. To elucidate their respective brain anatomical location, we deconvoluted the BrainSpan Atlas expression data. This analysis showed high expression of the majority of candidate genes in microglia-dense regions (Amygdala, Hippocampus, Striatum) in the postnatal brain. Furthermore, to decipher their localized expression across brain ages, we constructed a developmental dynamics map (DDM) comprising extensive gene expression profiles throughout prenatal to postnatal stages, which resulted in the discovery of novel microglia-specific gene signatures. One of the interesting readout from DDM is that all the microglia-dense regions exhibit dynamic regulation of few genes at 37 post conception week (pcw), the transition period between pre- and postnatal stages. To validate these findings and correlate them as potential biomarkers, we analyzed the expression of corresponding proteins in hESC-derived human microglia precursors. The cultured microglial precursors showed expression of Pentraxin 3 (*PTX3*) and *SPHK1* as well as several known markers like *CD68*, Allograft inflammatory factor 1 (*AIF1*/*IBA1*). In summary, this study has furnished critical insights into microglia dynamics across human brain ages and cataloged potential transcriptomic fingerprints that can be further exploited for designing novel neurotherapeutics.

Keywords: *SPHK1*, age, microglia stem cell-like progenitors, *PTX3*, transcriptomic analysis, amygdala, hippocampus, striatum

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INTRODUCTION

The complex human brain is composed of neurons embedded in a framework of glial cells (astrocytes, oligodendrocytes, and microglia) and blood vessels. Microglial cells, the third and highly motile type of glial cell populating the brain were first identified and simultaneously reported by F. Robertson and F. Nissl, who first gave them the name "Staebchenzellen" based on their rod-like nuclei shape (1, 2). Later, these cells were renamed as "microglia" by Pio del Rio-Hortega, who further characterized and delimited them from other glial types (3). It is noteworthy that the primary role of microglia is to dynamically monitor CNS-invading threats. Studies have proven that microglia play critical functions like maintenance of neuronal development by continuous patrolling of active neurons, scavenging dead neurons and dendritic spine pruning under normal physiological conditions (4–6). Abnormal microglial activities can either under-prune or over-trim the dendritic spines leading to progressive neurodevelopmental defects (5). Besides this, these brain scavengers can play multidimensional roles in maintaining brain homeostasis. One of the newly discovered roles suggests that microglia regulates the number of healthy neural precursor cells (NPCs) in adult brain (7, 8). First, microglia control the number of NPCs by selectively colonizing near proliferative NPC zones and phagocytizing the neural precursors *in utero*, and second, the microglial activity gets regulated in the brain by NPC-derived chemokines, such as *VEGF*, as shown by increased microglial density surrounding NPC pool (9, 10). Notably, this NPC-microglia crosstalk is most active during neuroinflammation, as microglia is one of the primary innate immunoeffector cells (10, 11). They usually accumulate around degenerating neurons, leading to the obvious misconception of causing detrimental effects. Albeit monumental efforts made toward understanding microglia dynamics in the developing brain, knowledge regarding their cellular localization, self-renewal/expansion property, and other stage-specific roles is still in its infancy.

Origin and continual existence of microglia throughout human brain span are being currently investigated. Interestingly, microglial ontogeny describes three lines of opinions. This includes its emergence either during the embryonic day (E7.0–E10.5) from yolk sac (YS) (12–15) or their infiltration during diseased conditions from bone-marrow (16). There is also a third view which suggests that microglia is of neuroectodermal origin, derived either from glioblasts or from the germinal matrix (17). In a recent study, Tay et al. established an *in vivo* fate mapping system and studied proliferation of microglia in the healthy and diseased brain which demonstrated a context-dependent growth pattern of microglia under normal physiological conditions and selective clonal expansion during disease states (18).

The burning questions of the hour deal with life-long inhabitation and maintenance of microglia in the human brain. Answers to these will shed light underlying disease onset and manifestations in several neurological diseases including Alzheimer's disease and Parkinson's disease (AD and PD). One of the challenges in microglial biology is to track their expression and activity throughout the brain development (fetal to adult and aged brain stages). Till date, hardly any study exists that has shed light on *in silico* or *in vivo* tracking of the microglial population (19). Specifically, microglial age and variance over brain regions can be the guidance cue to understanding onset and progression of neurodegenerative disorders (20–23). To track microglia-specific biomarker expression, their localization, and generation of a regulatory dynamic map, this study presents an integrated meta-analysis approach wherein, we analyzed the differentially expressed microglia-specific gene subsets using available data repositories. This led to a unique catalog of genes (27) which are dynamically/consistently expressed in the microglial population during different stages of brain development. For the first time, we have employed a novel strategy for *in silico* monitoring of microglial gene signatures to decipher their developmental dynamics map (DDM). Interestingly, protein–protein interaction network analysis revealed six different protein clusters, which exposed primary interacting partners like Tissue factor (coagulation) protein family, Retinoid X receptor gamma signaling linked *MGST* protein family, ATP-dependent protein family, and hippocampal BMP signaling molecules like *BMP4*. Furthermore, region specificity of the above candidates was evaluated using Allen Brain Atlas's (ABA) BrainSpan which revealed localized expression of several genes in microglia-centric regions. Finally, to validate the emergence of early/mature microglial signatures *in vitro*, we induced hESC-derived microglial differentiation using an in-house protocol. In conclusion, the study has validated the expression of stage-specific markers as well as presented an updated catalog of potential age-specific microglial biomarkers, which can be used as future prognostic biomarkers. The overall strategy adopted for *in silico* analyses has been compiled (**Figure 1**).

MATERIALS AND METHODS

Literature Survey

We utilized repositories including NCBI Pubmed and HighWirePress to understand the prior literature linked to microglia biology. Other high-throughput data repositories like NCBI's Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) were scanned for biological sequence data specific to transcriptomics of human microglia.

High-Throughput Data Analysis

First, using available RNA-sequencing data set linked to microglia transcriptomics (24), we delineated the brain cell-type specific expression in accordance with different brain cell types namely, microglia, astrocytes, endothelium, oligodendrocytes, and neurons. Specifically, we evaluated the differential expression between Rest of the Brain Cells comprising astrocytes, endothelial cells, oligodendrocytes and neurons (ROBC), and microglial cell type. Log_2FoldChange (FC) values threshold of ± 1.5 was considered with a p-value significance of <0.05. This formed our *primary* data set comprising microglia cell type-specific genes. Our *secondary* data set was utilized to gauge the correlation of differential expression between brain cell types and differential expression of induced pluripotent stem cells (iPSCs) versus derived microglia. This data series deposited in GEO database contains human microglia-specific microarray Affy HuGene-2_0-st data (GSE78115) (25). Specifically, we considered the 12 samples (3 replicates each) derived from 2 different cell lines NCRM-5 and iNC-01 of iPSC origin. Using OLIGO package of R

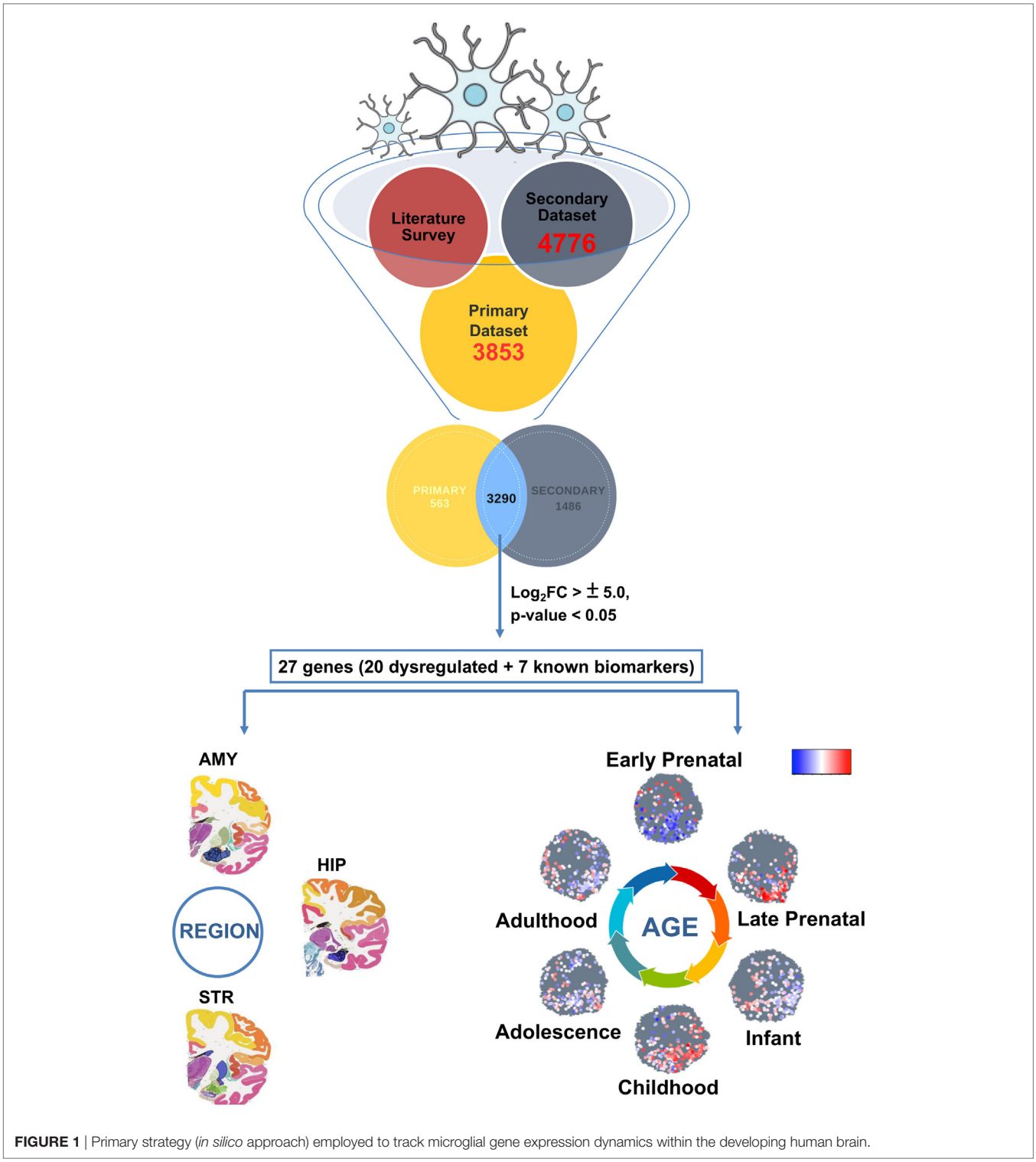

FIGURE 1 | Primary strategy (*in silico* approach) employed to track microglial gene expression dynamics within the developing human brain.

Bioconductor, we performed data analysis (including data normalization) to assess the differential expression pattern specific to human microglia with respect to iPSCs. The OLIGO package is freely available through Bioconductor website (26). Differential expression cutoff of ± 1.5 $\log_2$FC and p-value <0.05 was considered significant in all cases. The differentially expressed genes from both primary and secondary human microglia sample sets were combined to ensure minimal data loss. This integration yielded a common pool of microglial specific genes (3,290), which was further used for downstream analyses. Pearson correlation coefficient was calculated for the integrated gene list (3,290) using R. We further analyzed the expression patterning between the *primary* and *secondary* data sets using the top 20 (out of 3,290) up and downregulated genes, which were shortlisted on the basis

of *p*-value and log2FC values below 0.01 and above ±5.0, respectively. To this list, we also added a list of seven known biomarker genes specific to microglia for understanding their development expression dynamics. Taken together, the total list used for brain age and region-specific dynamics consisted of 27 genes.

Protein Classification and Pathway Analysis

We utilized Protein ANalysis THrough Evolutionary Relationships classification system and mapped the total differentially expressed list of 3,290 genes to various protein classes for elucidating their functional significance (27). For pathway analysis, we inputted the same gene list to Consensus PathDB-human to identify their key associative pathways (28). The Bonferroni corrected *p*-value cutoff <0.01 was applied for both analyses.

Gene Set Enrichment Analysis

In order to ascertain significant gene sets in our differentially expressed gene list, we implemented Gene Set Enrichment analysis using the Broad Institute GSEA v2.07 software,[1] the molecular signatures database,[2] and the C5: GO gene sets database, comprised of 1,454 gene sets named by genes and respective GO terms.[3]

Protein–Protein Interaction Network Analysis

The differentially expressed gene list (3290) was mapped to STRING for whole proteome network analysis. Furthermore, the shortlisted microglia-specific gene list (27) was also analyzed for protein partner associations using STRING (29). Primarily, using all the top upregulated and downregulated protein clusters, a single protein–protein network was constructed. A stringent cutoff of 0.7 for edge/interaction score was considered for building the primary protein–protein interaction network without clustering and no more than 20 primary interactor proteins. This combined confidence score is calculated on the basis of various parameters including phylogenetic co-occurrence, gene fusion, homology, co-expression, experimentally determined interaction, and neighborhood on any chromosome. The graph was primarily constructed according to interaction/edge score while specifying the source and target nodes. All networks were visualized using Cytoscape 3.4.0 (30).

Brain Region Expression Mapping

The Allen Brain Atlas contains an extensive catalog of human brain expression data (Microarray and RNA-Seq) with region specificity (31, 32). We collected the raw data of the developmental transcriptome from Allen Brain Atlas and segregated the data into different ages from prenatal to postnatal (adult) brain (31). Here, we mapped the top gene set of 27 genes onto all the prenatal and postnatal brain regions, with a special emphasis on human microglia-enriched regions like Hippocampus, Striatum, and Amygdala. Other than these genes, we also show the expression of known microglial marker genes like *TMEM119*, *P2RY12*, Phospholipase D4 (*PLD4*), *AIF1*, *PTPRC1*, Sphingosine kinase 1 (*SPHK1*), *PTX3*, and G protein-coupled receptor 34 (*GPR34*).

Age-Wise Dynamics

To assess the microglia dynamics throughout the age (pre- and postnatal) of a human being, we utilized the human developmental transcriptome data (BrainSpan) deposited in the Allen Brain database (33, 34). Replicate samples for each developmental stage (prenatal or postnatal) were considered for analysis. The age groups ranged from prenatal stages, including 14th postconception week (pcw), 22 pcw to 40 years of age. Specifically, for each microglia-enriched region of the brain (hippocampus, striatum, and amygdala), we constructed individual graphs for age-wise DDM of potential and known microglia-specific markers which included shortlisted gene set of 27, containing 20 (p-value < 0.01, $\log_2$FC > ±5.0) and 7 known markers. Following this, correlation analysis was performed using the same gene list as input to find correlations among the human ages spanning prenatal stages like 12 pcw, 37 pcw to postnatal stages, such as 1, 15–19, and 40 years of age. Pearson correlation matrices or correlograms were constructed and visualized using the Corrplot package of R Bioconductor suite. All graphs have been made using RStudio 1.0.153 (35).

Culturing of Human Embryonic Stem Cells (H9)

Human embryonic stem cells (h9-hESC, NIH approved) were obtained from human embryonic stem cell core, Baylor College of Medicine. H9 clones were propagated based on feeder-free culture system, using human ESC-qualified Matrix, BD Matrigel (BD Biosciences, San Jose, CA, USA), and grown in mTeSR-1 complete media supplements (Stemcell Technologies, Vancouver, BC, Canada) at 37°C and 5% CO_2. Clones were maintained and expanded according to manufacturer's protocol, in accordance with NIH guidelines. Complete media was changed every day and cells were sub-cultured using Dispase (Stemcell Technologies, Vancouver, BC, Canada) every 5–6 days. During each stage of subculture, uniform H9 colonies were collected without any visible differentiation.

Embryonic Body (EB) Formation

To induce the formation of EBs or neuroectodermal spheres, the H9 colonies with 70% confluency were used. To achieve neural induction, H9 colonies were incubated in neural induction me-dium [NIM; DMEM/F12: Neurobasal (1:1), 2% B27, 1% N2 (Invitrogen)] for almost 24 h and dissociated into small clumps by manual scraping. Small clumps were further grown as suspension culture with NIM in non-coated bacterial Petri dishes for a period of 4 days as exactly described by Cho et al. (36). 50% of the media were replaced in each of the EB-containing Petri dishes at a timely interval of 48 h.

Microglial Differentiation and Generation of Microglia Precursors From hESC

To induce microglial differentiation in culture from H9 derived EBs and obtain human microglial precursors *in vitro*, we used an in-house standardized protocol (unpublished), modified based on an earlier established protocol (37). In brief, the whole process

[1] http://www.broadinstitute.org/gsea (Accessed: July 17, 2017).
[2] http://www.broadinstitute.org/gsea/msigdb (Accessed: July 17, 2017).
[3] www.geneontology.org (Accessed: July 17, 2017).

goes through a long-term culture (approximately 8 weeks) covering four major steps, (i) formation of hESC (H9) derived EBs, (ii) neuroectodermal lineage commitment, (iii) microglial differentiation, and (iv) formation of induced microglia precursors (iMPs), as suggested in the previous article by Beutner et al. (37). We have used stage-specific conditioned media supplements as suggested by Beutner et al., including differentiation media (DIFF), Insulin/Transferrin/Selenite/Fibronectin media ($ITSF_n$) and N2 Media. However, we have introduced several modifications during the stage-specific progression of microglial differentiation. These include the following: (1) Usage of NIM media till 5 Days *in vitro* (DIV) for maintaining EBs and then switching them to $ITSF_n$ on sixth day, (2) addition of human stem cell factor (hSCF, Peprotech, USA) and basic fibroblast growth factor (Peprotech) in culture from DIV 7 to DIV 14 with concentrations of 50 and 25 ng/ml, respectively, (3) treatment of cultures from DIV 14 to DIV 21 with hSCF, hIL3, and human granulocyte macrophage stimulating factor (hGM-CSF, Peprotech) with concentrations of 50 ng/ml for both hSCF and hIL3, and 25 ng/ml for hGM-CSF, and finally (4) addition of only hGM-CSF from DIV 21 onward till DIV 40 and furthermore.

Immunocytochemistry/ Immunofluorescence Analysis

Cells from different stages of microglia induction and differentiation were fixed for performing immunocytochemistry and immunofluorescence-based analyses using primary antibodies and fluorophore-conjugated secondary antibodies. Detailed information related to fixation, antibodies, dilution, and image analysis are listed within Data sheet 1 (Supplementary Table 1) in Supplementary Material.

Statistical Analyses

Differential expression cutoff of ± 1.5 $\log_2$FC and p-value < 0.05 was considered significant unless specified. Bonferroni corrected p-value cutoff $= 0.01$ was applied in protein classification and pathway analyses. At least triplicate donors were considered for each developmental stage (prenatal or postnatal) for age/region analyses. In case of the *in vitro* experiments, we have maintained biological replicate of $n = 3$.

RESULTS

Cell-Type Specific Gene Set Enrichment Analysis of Human Microglia

The complete GEO and SRA high-throughput databases were scanned to identify microglia-specific data sets. Out of this, two data sets were shortlisted for further in-depth *in silico* analyses. Initially, we analyzed the *primary* data set, i.e., the total gene set from the cell-type specific data comprised of significantly expressed genes in ROBC versus microglia (**Figure 2A**). A total of 3,853 genes were differentially expressed, with a range comprising 6.0 $\log_2$FC as the maximum expression to -9.4 $\log_2$FC as the minimum expression. The samples of various cellular origins were tested for homogeneity *via* 2D-Principal Component Analysis which resulted in noticeable differences in microglia sample cluster from other cell types in Principal components, PC 1 and PC 2 (**Figure 2B**). We specifically considered the *primary data set* to be the cell-type data to ensure elimination of non-microglia related noise in the human brain. Furthermore, we identified known biomarkers including *SPHK1*, *PLD4*, *TMEM119*, and *CD68*, expressed in the range of 2–6 FC and used them as a positive control for *in silico* tracking of microglia.

Sphingosine kinase 1 is a known biomarker for quiescent neural stem cell (qNSC) pool and the key enzyme responsible for phosphorylation of sphingosine to sphingosine-1-phosphate (*S1P*) (38). It has been also seen to play an important role in the regulation of proinflammatory cytokines in activated microglia (39). Purinergic receptor 12 (*P2RY12*) is known to suppress microglial process motility and delay in closure of blood–brain barrier (BBB) (40). *P2RY12*-mediated microglial cell activation contributes to the rapid closure of BBB small leaks, by aggregation of microglial cells at the injury site. Expression of Phospholipase D4 (*PLD4*) and its localization changes are correlated with the activation state of microglia (41, 42). This transmembrane glycoprotein localized in the endoplasmic reticulum and Golgi apparatus is primarily seen to play an important role in microglia dynamics during early postnatal brain development. Transmembrane protein 119 (*TMEM119*) is an established, reliable microglial marker that discriminates resident microglia from blood-derived macrophages in the human brain (43). A glia-derived Pentraxin 3 (*PTX3*) expressed in the microglial secretome, has been seen to modulate phagocytic functions of microglia having crucial implications in the regulation of microglial activity in brain diseases (44).

The *secondary* data set was a redundant list of total 4,776 genes which was seen to be differentially expressed among 2 human microarray data sets. This data set contains six induced microglia samples derived from human iPSCs (25). We integrated the differentially expressed microglia-specific data enriched from both *primary* and *secondary* data sets containing 3,853 and 4,776 genes, respectively. This meta-analysis yielded a common pool of microglial specific genes (3,290) which was further used for downstream analyses (**Figure 1**).

Pathway Mapping and Gene Set Enrichment Analysis

The readout of gene candidates (3,290) was used to target enriched pathways. Among the candidate pathways, GPCR, Glycerophospholipid, NOTCH1, RAP1, glycosaminoglycan metabolism, VEGFR signaling, etc. were significant (**Figure 2C**). This analysis also demonstrated enrichment of 278 out of 3,290 genes within the immune system pathway, suggesting their potential roles in microglia functioning or immune surveillance (**Figure 2C**).

The list of significantly expressed genes (3,290) was used for gene/protein type-based clustering. This analysis revealed 25 crucial protein classes that majorly comprise of 10.7% enzyme regulators, followed by 9.9% nucleic acid binding proteins, and enzymes like transferases, hydrolases, transcription factors, and signaling molecules (Figure S1 in Supplementary Material). The other segments included cell junction, immunity/defense and extracellular matrix proteins containing 28, 40 and 46 proteins, respectively. These classes acted as guidance cues for

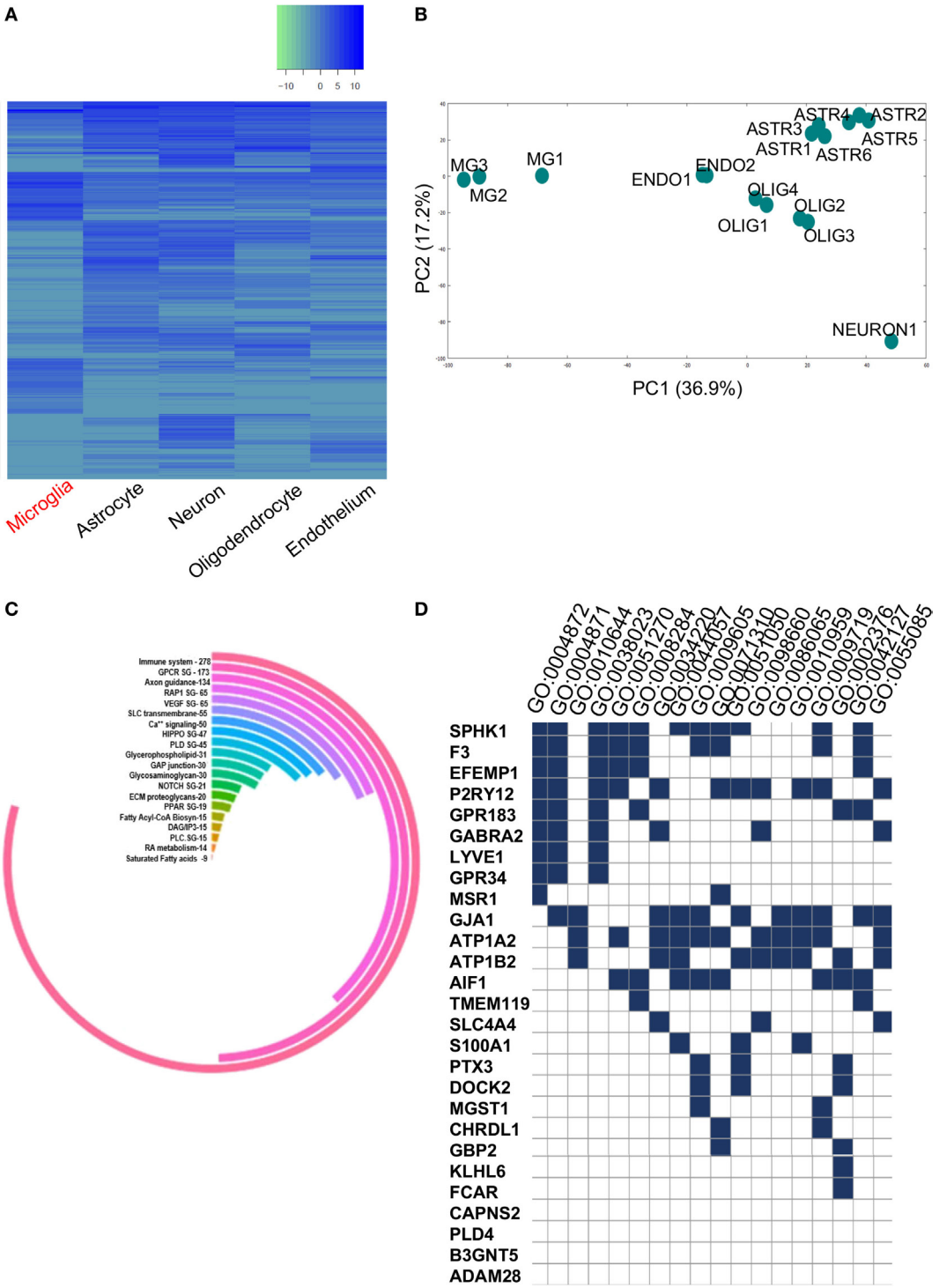

FIGURE 2 | **(A)** Heatmap representing significantly expressed upregulated and downregulated gene profiles of various brain cell types. **(B)** Principal component analysis plot showing homogeneity within each cell type and diversity among the five brain cell types namely, microglia, endothelial cells, oligodendrocytes, neurons, and astrocytes. **(C)** Circular plot showing pathway analysis of total dysregulated gene list (3,290) between ROBC and microglia. **(D)** GSE Analysis showing enriched GO BP/MF terms ($p < 0.05$) in 27 shortlisted genes overlapping between primary and secondary datasets.

the protein–protein based network analysis as well as pathway mapping and exposed many critical clusters of gene regulatory elements. We also performed GSEA on critically altered gene set of 20 dysregulated genes and 7 known microglia-specific markers to find critical GO terms like Immune System process, Receptor activity, Regulation of cell proliferation which further

substantiated the data (**Figure 2D**; Supplementary Table 2 in Supplementary Material). The aforementioned shortlisted gene set (27) was used for further analyses.

Correlation Analysis of Microglial Biomarkers Among the Data Sets

To establish correlations among the *primary* and *secondary* data sets, we calculated the Pearson correlation coefficient for the common gene pool between the two data sets (3,290), which resulted in a value of −0.22 (**Figures 3A,B**). First, the primary and secondary datasets were analyzed for the similarity in expression of shortlisted gene set comprising 27 (20 novel + 7 known) genes (**Figure 3C**). Within the upregulated gene set, all these biomarker genes excluding *GPR34* demonstrated similarity in expression when compared to the *secondary* data set [iPSC derived microglia (iMG) data] (**Figure 3B**). To highlight, top upregulated genes, including Dedicator of cytokinesis 2 (*DOCK2*), Guanylate binding protein 2 (*GBP2*), A disintegrin and metalloproteinase 28 (*ADAM28*), G Protein-coupled receptor 183 (*GPR183*), Calpain small subunit 2 (*CAPNS2*), and Fc-α receptor mediator (FCAR), were found to be consistently expressed in primary and secondary data set.

Further literature mining of this top altered gene set (27) substantiated their mechanistic roles relevant to neuroinflammation and neurotoxicity. Among these, *DOCK2*, an atypical Rho guanine nucleotide exchange factor (GEF) for *RAC* and/or *CDC42* GTPases are known to regulate phagocytic activity, cytokine release, and paracrine neurotoxicity in microglia (45). In one of the microglia-mediated neuroinflammatory cascades, the *DOCK2-RAC1* pathway was seen to be elicited by S1P through an integrin-dependent association (46). Precisely, S1P which is the phosphorylated end-product of *SPHK1* is known to regulate immune cell trafficking upon binding to G-coupled receptors (*GPRs*) (47). Interestingly, we found fivefold expression of a novel marker *GPR183* in microglia whose role is yet to be elucidated. *DOCK2* is directly regulated by the Prostaglandin E2 receptor (*EP2*) and has also been linked with AD pathophysiology (45).

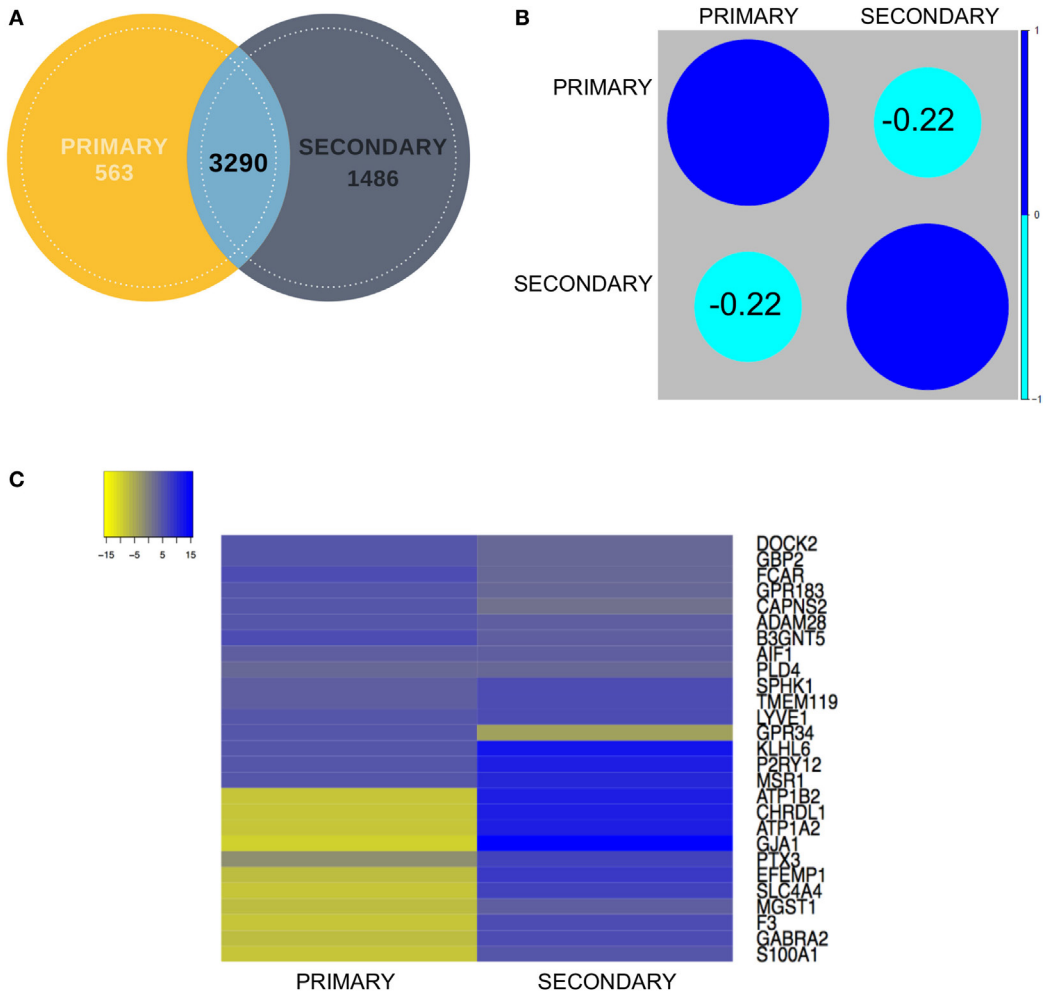

FIGURE 3 | **(A)** Venn diagram showing contribution of each dataset (primary and secondary) to the overlapping gene pool. **(B)** Correlation analysis between the primary and secondary datasets showed mildly negative correlation. **(C)** Representative expression heatmap between the final shortlisted 27 genes has been elaborated in the expression map for in-depth correlation analysis.

Concurrent evidence involving Prostaglandin signaling in microglia also suggested that balanced induction of Peroxisome proliferator-activated receptor gamma (*PPARG)* by *LPS/IL13* or activation of *EP2* can lead to neuroprotection or death of microglia *via* calpain-dependent pathway (48). Our data also validated the elevated expression of *PPARG* as well as its downstream target calpain associated genes like *CAPNS2* in the microglial pool versus the other cell types.

Another immunoeffector molecule, *FCAR* (*CD89*) was seen to be highly expressed among the top 20 altered genes in the microglial population, which also corroborated by a recent report by Galatro et al. (49). Previous reports suggest that human microglial cells expressing truncated IRF3 show decreased expression of *GBP2*, thus proposing a possible link of this protein to *TLR3/4*-dependent neuroinflammation. Our study has shown another important marker of the microglial cell type known to be involved in CNS invasion, i.e., *ADAM28*, a member of the metalloproteinase family (50). Another enriched marker gene *N*-acetylglucosaminyltransferase *B3GNT5* was seen to be a prominent player in the expression of lactoseries sulfoglucuronylglycolipids within glycosphingolipid pathway during the prenatal brain develop-ment (51). High expression of Kelch-like family member 6 (*KLHL6*) was also detected in the microglial pool, which is supported by previous AD mice model-based studies linked it to AD pathology (52). We also found an interesting factor known as macrophage scavenger receptor 1 (*MSR1*) involved in the phagocytic machinery of microglia (53).

Contrary to this, in case of top 10 downregulated genes, the expression values for the *secondary* data set were found to be non-correlative to the primary data set (ROBC vs. microglia) (**Figure 3C**). Microsomal Glutathione S-Transferase 1 (*MGST1)*, a regulator of Retinoid X receptor gamma signaling during remyelination showed non-correlative expression between the two data sets (54). Genes like S100 Calcium Binding Protein A1 (*S100A1*) (55) and Gamma-aminobutyric acid receptor subunit alpha-2 (*GABRA2*) (24, 56) which are known to be highly expressed in astrocytes were also seen to be enriched in the secondary data set and repressed in the primary data set, thus justifying the need to enrich a pure microglia cell type marker-enriched population for *in silico* and *in vivo* tracking. We also detected a novel gene EGF Containing Fibulin Like Extracellular Matrix Protein 1 (*EFEMP1*), a fibulin glycoprotein family member which was found to be downregulated in ROBC versus microglia. This was earlier shown to be upregulated in malignant gliomas and age-related macular degeneration (57). Another interesting observation suggested expression of several synaptosomal and synaptic receptor genes like *SLC4A4* and *GABRA2* significantly downregulated in the primary data set in coherence with the study by Ji et al. (58). This analysis sheds light on probable roles of the top 20 genes in other brain cell types as well as exposes a filtered population of markers linked to microglia function in the developing human brain.

Protein–Protein Interaction Network Analysis

To develop an in-depth understanding of the microglia-specific markers and their primary neighborhood protein partners, we constructed a primary protein-protein interaction network. Out of the input list of 3,290 genes, 994 coding proteins showed interactions with a confidence score above 0.70 (**Figure 4**). Network statistical analysis showed the Network centralization score to be 0.07 and clustering coefficient to be 0.423 with a total number of hub nodes to be 43.

Subset PPI networks were constructed based on the top 20 differentially expressed gene list in combination with the known biomarkers. This network exposed primary neighborhood interacting partners of *DOCK2*, *F3*, *GJA1*, *CHRDL1*, *MGST1*, and *ATP1A2* (**Figure 4**). Among these, one of the highly upregulated genes, *DOCK2* showed multiple interacting partnerships with known biomarkers like *SPHK1, PTPRC*, and *CD68*, which is substantiated by numerous connections (edges) and their respective confidence scores (**Figure 4**). *DOCK2* is also seen to be connected to *SPHK1* through *LYN*, and to *PTPRC*, and *CD68* through *FYN* (**Figure 4**). In a second cluster, we found *GJA1* to be interacting with *CDH2* and *TJP1*. The *GJA1* mutation has been seen to cause Oculodentodigital dysplasia, an autosomal dominant disorder, with high penetrance in intra- and interfamilial phenotypes (59). In another cluster, F3 or thrombosis factor 3 was prominently associated with its own family members (**Figure 4**). F3 gene encodes coagulation factor 3 which is a membrane-bound glycoprotein is part of the Tissue factor family. Although no specific links were found between *F3* and brain development, it has been shown to be regulated during brain neoplasms (60) or in the innate immune cells (61, 62). Based on GSEA, correlation, and PPI analysis, we conclude that *DOCK2* acts as a molecular hub for channelized signaling in microglia-based neuroprotection, neuroinflammation, and neurological diseases, including AD and PD, Autism spectrum disorders, and schizophrenia (63). This newly discovered microglia-specific regulator is known to be associated with disease pathology of AD patients independent of COX signaling (45).

Trends in Region-Specificity for Potential, Novel Microglia Signatures

Cellular localization of microglia is still debatable in human brain development. Notably, the prenatal human brain displays smaller brain size with a higher number of sub-regions (26), whereas the postnatal brain contains 18 developed sub-regions. Microglia population in the healthy brain is known to be residing primarily in hippocampus, amygdala, substantia nigra, and striatum. However, developmental tracking of human microglial activity in these regions is still elusive. To elucidate the region-specific dynamic regulation of microglial markers, we analyzed the distribution of the identified *in silico* candidates. Our data revealed that in the prenatal and postnatal brain regions, the expression profiles of selected dysregulated gene set (20 + 7) was dynamically regulated as seen by their variable expression across sub-regions. Understanding this variability in their expression pattern would be crucial for building the microglial dynamic map in human brain development.

In the prenatal brain, the expression levels of known biomarker genes like *PLD4*, *SPHK1* were high in regions of importance like caudal ganglionic eminence, medial ganglionic eminence, lateral

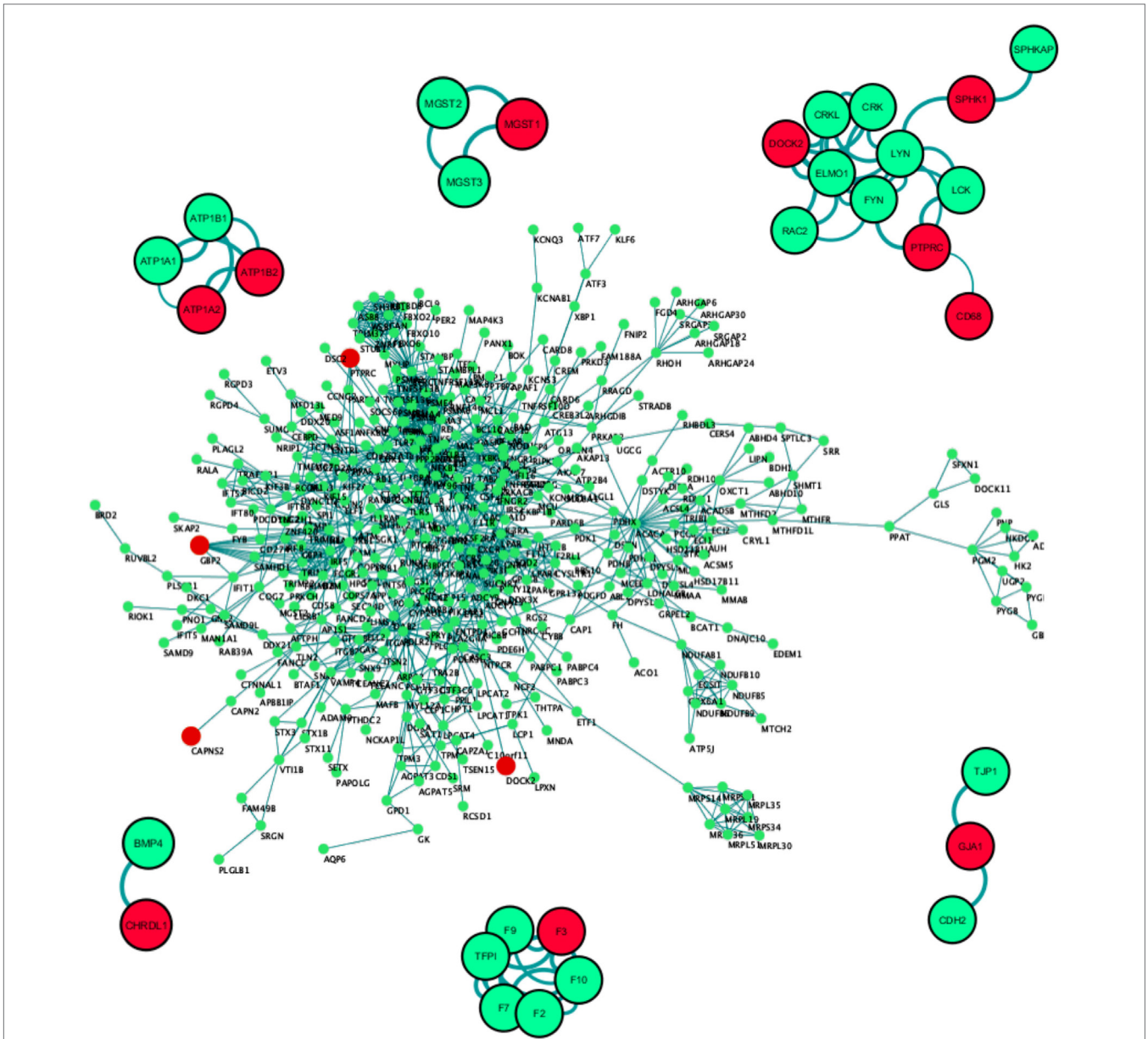

FIGURE 4 | PPI network construction of dysregulated proteome coded by 3,290 genes. Only 994 out of 3,290 genes passed the threshold interaction score of 0.70. The second representative protein–protein interaction network of the top differentially expressed gene set (27 genes) showed 11 out of 27 proteins coded by differential expressed genes to be actively interacting with each other or with varied protein partners. Red: differentially expressed gene in the primary data set, green: interacting partner, edge indicates the source to target node strength. Combined interaction score > 0.70, No. of first level interactors = 20.

ganglionic eminence and lower in regions like posteroinferior parietal cortex, inferolateral temporal cortex, medial frontal cortex (MFC) (**Figure 5A**). However, *GPCR* associated genes like *GPR183* and *GPR34*, and EFEMP1 were seen to be highly expressed only in the primary visual cortex (V1C) region in the prenatal brain (**Figure 5A**). To highlight, *EFEMP1* mutations are associated with Doyne honeycomb retinal dystrophy, an autosomal dominant disorder in which there are drusen (lipid) deposits in the macula eventually leading to vision loss (64, 65). In the postnatal brain, genes such as *GBP2*, astrocyte marker *S100A1* and *MGST1* are highly expressed in the cerebellar cortex region, while genes like *GPR183*, *EFEMP1*, and *GPR34* are only expressed in MFC (**Figure 5B**).

In summary, this analysis revealed consistent high expression of 14 out of 27 microglial markers in regions like hippocampus (HIP), amygdala (AMY), and striatum (STR) during postnatal development (**Figure 5B**). However, all of these 14 genes are low in expression throughout prenatal development (**Figure 5A**). Interestingly, amygdaloid complex (AMY) of the prenatal brain showed very low expression of all 27 genes, whereas the same

region showed pronounced expression of numerous genes, like *P2RY12*, *DOCK2*, *MSR1*, *B3GNT5*, *AIF1*, etc. during postnatal development (**Figure 5B**). Overall, this analysis has provided region-based developmental tracking of microglial expression dynamics in the human brain.

Age-Specific Correlation of Microglial Signatures During Brain Development

Ontogeny, renewal, and maintenance of microglial population have long been debated. In this direction, we decided to correlate region-specific expression of microglial markers to developmental stages of the human brain. The ABA developmental transcriptome data yielded a humungous gene pool constituting age-wise microglial marker expression specifically in AMY, HIP and STR regions. We, further, focused on the gene regulation status of top 10 upregulated and downregulated gene sets across brain ages encompassing prenatal (8–37 pcw) and postnatal (1–40 years). Furthermore, we built correlation matrices between several important ages, including 13 and 37 pcw, marking the prenatal ages, and 1, 8, 15, 19, and 40 years indicating infant to the adult brain.

The correlation matrices with a correlation coefficient scale (−1, 1) for all three brain regions showed positive correlation at varying degrees. The representative correlogram for prenatal stages of *HIP* showed moderately positive correlation ($R = 0.65$) between 13 and 37 pcw and aged brain stage of 40 years ($R = 0.70$) (Figure S2 in Supplementary Material). Interestingly, a very strong

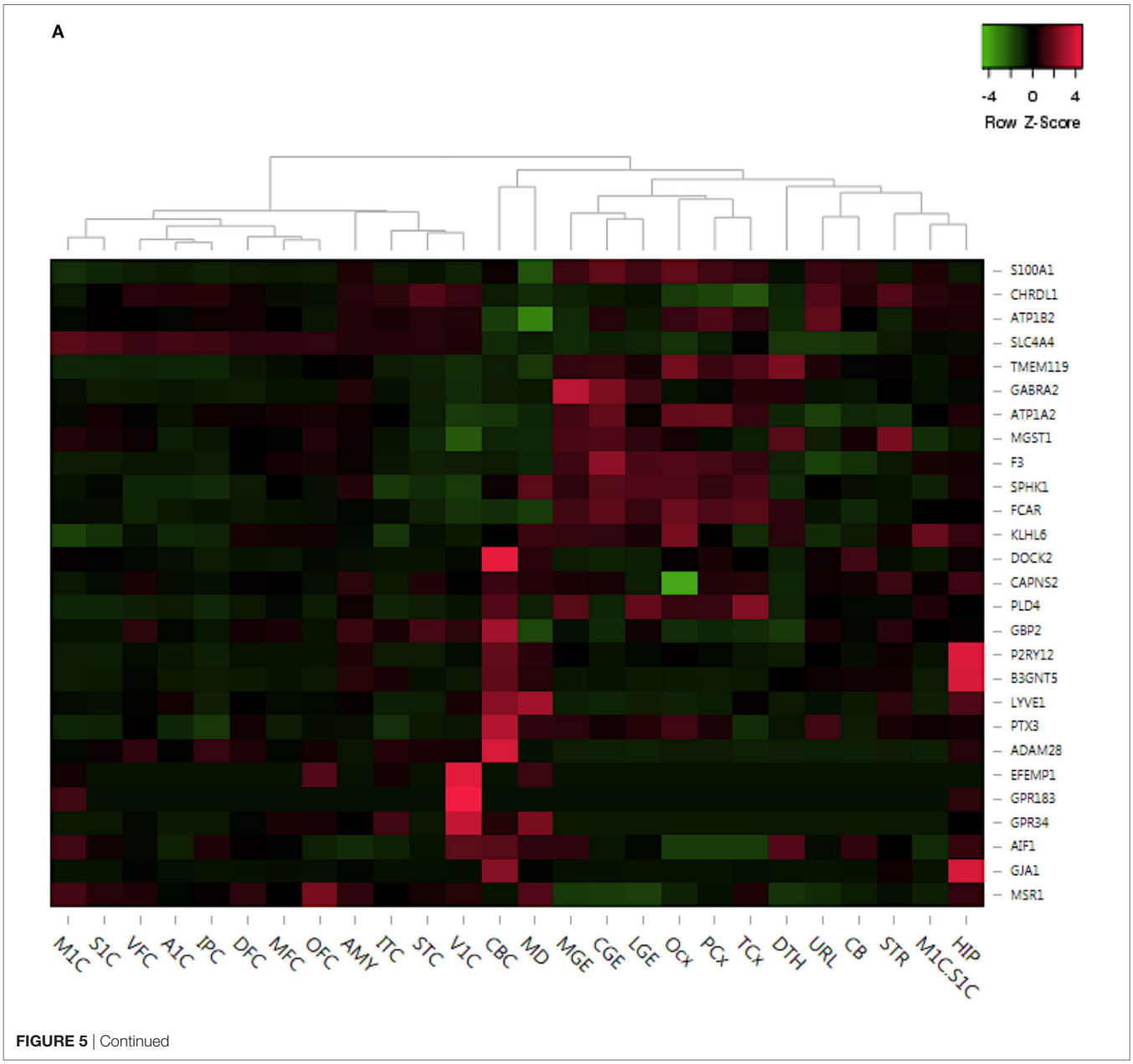

FIGURE 5 | Continued

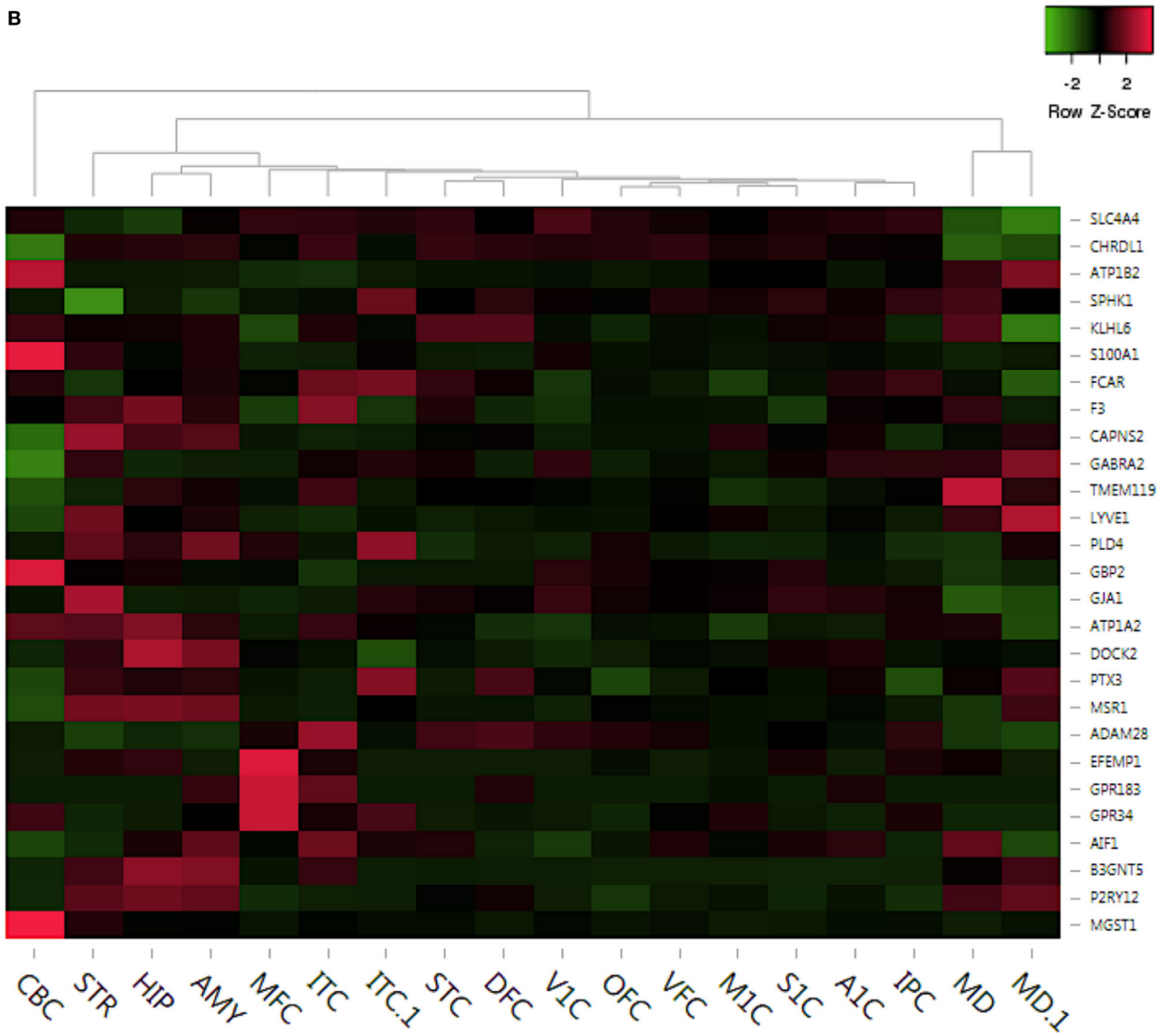

FIGURE 5 | Delineation of top dysregulated gene expression profiles across crucial brain anatomic regions associated to neurodevelopment. **(A)** Prenatal region-wise expression of the top upregulated and downregulated genes. **(B)** Postnatal region-wise expression of the top upregulated and downregulated genes. Abbreviations: DFC, dorsolateral prefrontal cortex; VFC, ventrolateral prefrontal cortex; MFC, anterior (rostral) cingulate (medial frontal cortex); OFC, orbital frontal cortex; M1C, primary motor cortex (M1); S1C, primary somatosensory cortex (S1); IPC, posteroinferior (ventral) parietal cortex; A1C, primary auditory cortex (core); STC, posterior (caudal) superior temporal cortex (Tac); ITC, inferolateral temporal cortex (Tev); V1C, primary visual cortex (striate cortex, V1); HIP, hippocampus (hippocampal formation); AMY, amygdaloid complex; STR, striatum; MD, mediodorsal nucleus of thalamus; CBC, cerebellar cortex.

correlation was also noticed between the early adult ages of 8 years and later ages like 18 and 40 years ($R = 0.90$) in the *HIP* region. Respective correlogram for *STR* displayed increasing correlation (0.77) between 13 and 37 pcw and all adult ages, and notably, highest correlation score was observed between 19 and 40 years of age ($R = 0.97$) (Figure S2 in Supplementary Material). In case of *AMY*, we noticed very strong correlation between the postnatal ages of 1, 5, 15, and 40 years ($R = 0.88$). However, a moderate correlation was observed among 13 and 37 pcw ($R = 0.67$), and 13 pcw and 40 years stages in case of *AMY* region (Figure S2 in Supplementary Material).

Upregulated Genes in Age-Wise Distribution

In the *HIP*, known biomarker genes like *AIF1* was found to be consistently high ($\log_2$FPKM ~ 5.0), while the expression levels of *SPHK1* and *GBP2* remained low ($\log_2$FPKM < 0). In *STR*, *GBP2* was highly expressed, while *AIF1* and *SPHK1* were low in expression ($\log_2$FPKM < 0). In *AMY*, *AIF1* and *SPHK1* showed consistently high expression ($\log_2$FPKM ~ 3.5, 3.0), while GBP2 remained low in expression throughout prenatal to postnatal brain developmental stages ($\log_2$FPKM < 0). In case of the top upregulated genes (10), variation in gene expression was strongly evident (**Figure 6**).

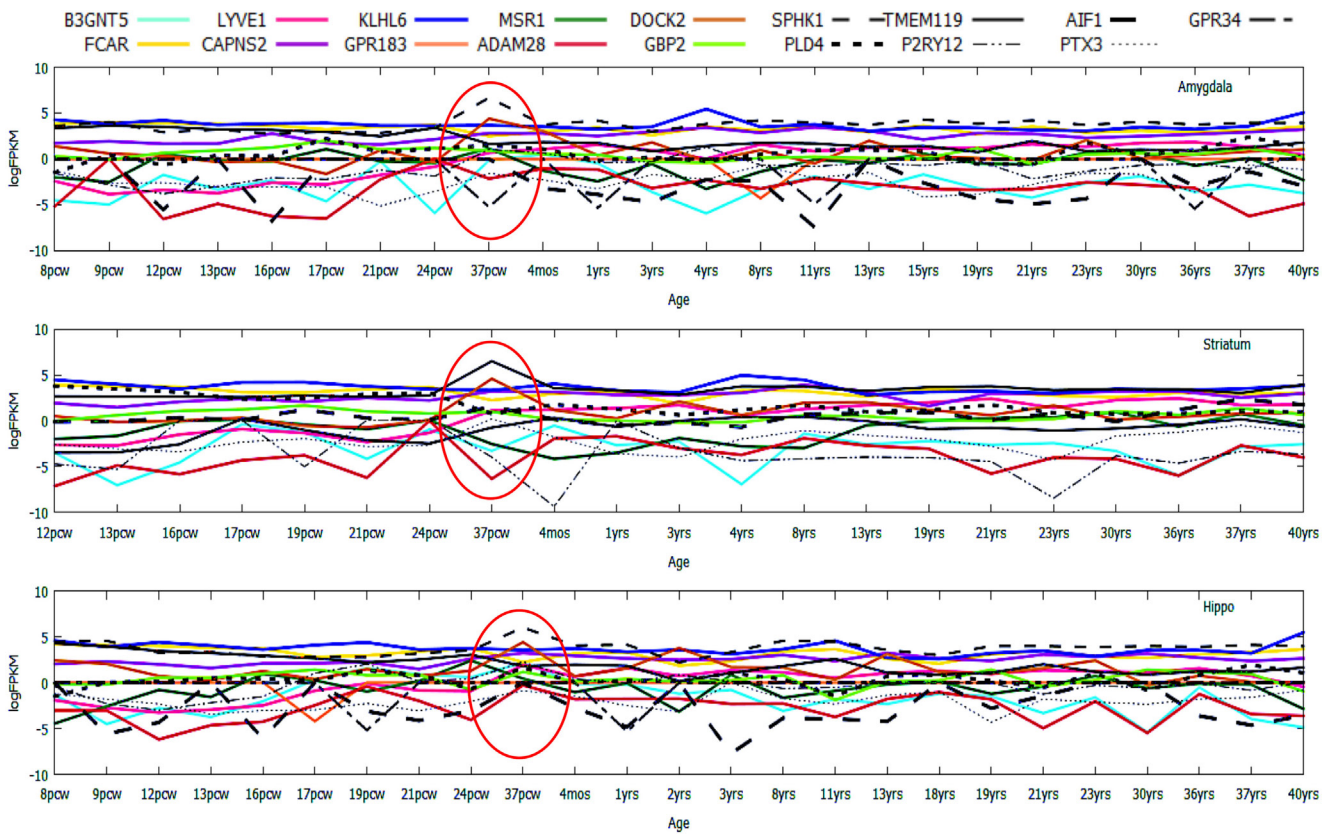

FIGURE 6 | Age-wise expression dynamics of highly upregulated genes across microglia-enriched regions of the human brain. Gene expression profiling of top 10 upregulated genes and known expressed biomarkers (17) in the developing/degenerating brain including prenatal stages like 9 pcw fetus and postnatal brain stages like 3 years to a grown adult 40-year-old brain.

Among the top regulated genes, Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (*LYVE1*), a type I integral membrane glycoprotein was found to be consistently repressed till the prenatal stages, followed by gradual increment in expression during postnatal stages (after 37 pcw) with minor inconsistencies in all the three brain regions (**Figure 6**). A supporting study from mice also showed similarity in the variation of *LYVE1* expression profiles in hippocampal and cerebellar regions (19). Consistent high expression of *FCAR* and *KLHL6* throughout pre- and postnatal development was distinct in all three aforementioned regions. The most inconsistently expressed genes across all the three regions included *DOCK2* and *GPR183* exhibiting a rapid decrease in expression at various ages like 12 pcw, 17 pcw, 24 pcw, 8 years, 21 years, 30 years, and 37 years suggestive of their dynamic roles in brain aging.

Downregulated Genes in Age-Wise Distribution

In case of the top downregulated gene set (10), a uniform pattern of expression was observed. In the *HIP*, all the genes were found to be consistently high in expression ($\log_2$FPKM ~ 4.5) throughout all the age groups excluding two inconsistently expressed genes, *GJA1* and *EFEMP1* (**Figure 7**). We also found that *EFEMP1* showed unaltered expression till the age of 19 years, followed by a sharp dip in expression at the ages of 21 and 40 years. On the other hand, *GJA1* showed prominent inconsistency with a gradual decline toward 13 pcw, increase at 17 pcw, sharp dip at the age of 4 months and finally, remained unaltered in the rest of the postnatal-adult ages. In *AMY*, all the genes showed consistently high expression ($\log_2$FPKM ~ 4.0), which then gradually decreased toward the postnatal stages ($\log_2$FPKM ~ 3.0) excluding F3 gene. Surprisingly, expression of *F3* was seen to be significantly low ($\log_2$FPKM < -2) during prenatal stages, with inconsistency in expression during postnatal ages (**Figure 7**). In *STR*, all genes except *EFEMP1*, *GJA1*, and *GABRA2* were seen to be continually expressed at a high level throughout all age groups. Especially, *GABRA2* showed sharp dips at 17 pcw, 37 pcw, and 3 years whereas, *GJA1* remained repressed ($\log_2$FPKM < 0) with gradual increment in expression during the postnatal stages ($\log_2$FPKM ~ 1.0) (**Figure 7**). Early pieces of evidences from *GJA1*/Connexin 43 null and conditional knock-out mouse brains revealed its strong regulatory role in overall brain development (66). The age-wise expression map (DDM) represented a set of gene fingerprints specific to microglia which consisted of seven genes, namely, *EFEMP1*, *GJA1*, *PTX3*, *KLHL6*, *SPHK1*, *FCAR*, and *CAPNS2* (**Figure 8A**). This gene set showed consistently altered expression (both up and downregulated) in all three

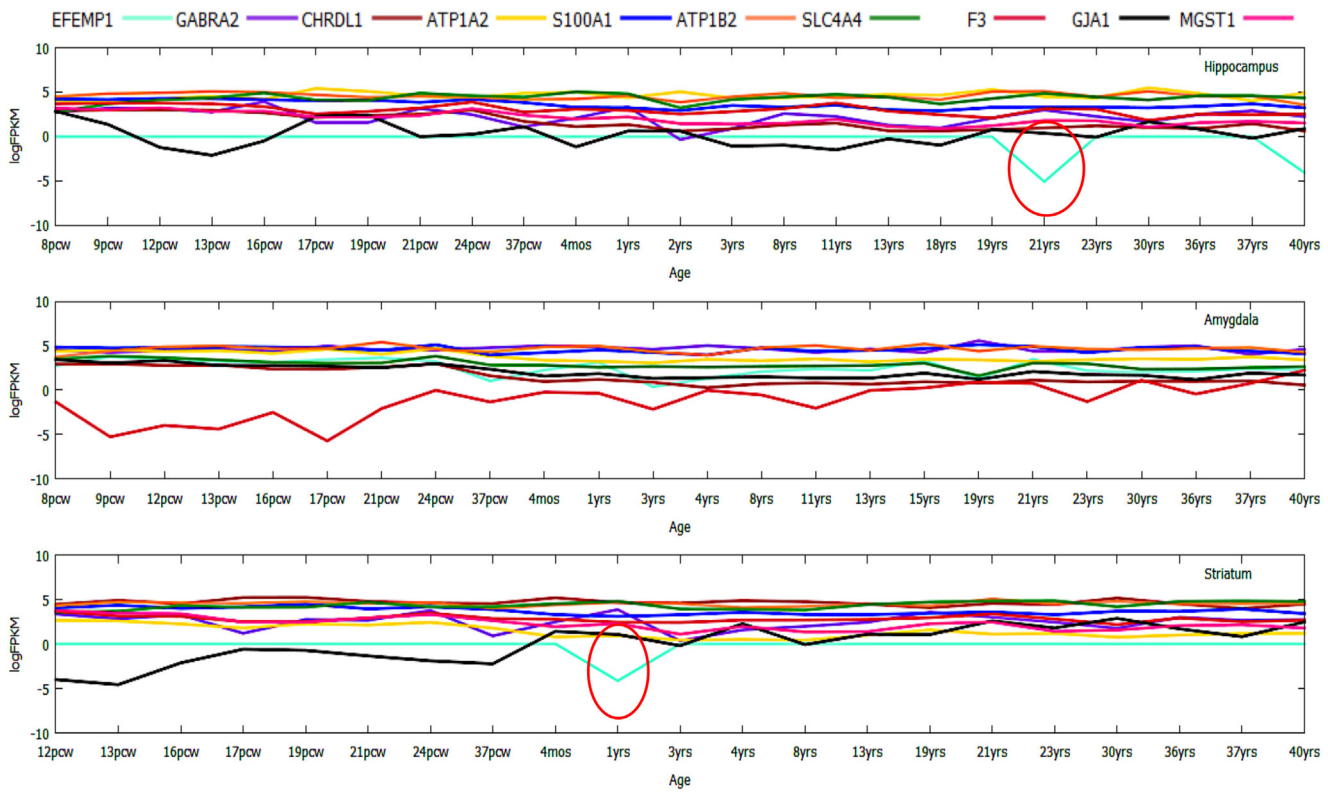

FIGURE 7 | Gene expression profiling of top 10 downregulated genes during prenatal to postnatal brain developmental stages displaying numerous inconsistently or dynamically expressed genes like *DOCK2*.

microglia-dense regions, across the human brain development. To highlight, DDM also revealed three most dynamically regulated genes namely, *DOCK2*, *LYVE1*, and *TMEM119* demonstrating an irregular pattern of expression across the brain ages (**Figure 8B**). These gene signatures can be further explored *in vitro* to elucidate their specific roles in microgliogenesis.

In Vitro Validation of Microglia-Specific Biomarkers in the Cellular Model of Microglia

To validate the emergence of microglia-specific biomarkers during brain development, we have established a cellular model of human microglia using an *in-house* standardized method. To generate human microglial precursors *in vitro*, hESCs were induced for microglial commitment using a long-term culture platform, which could be maintained for a period of 4 to 6 weeks. To authenticate the generation of microglial precursors *in vitro*, we have evaluated the stage-specific lineage commitment that comprised of (i) formation of EBs, (ii) neural induction, and (iii) generation of early and late microglial precursors using specific biomarkers. Details of the culture stages have been explained in the scheme (**Figure 9**). Interestingly, the *in silico* age dynamics study has projected *SPHK1* as a key marker to be consistently upregulated at 37 pcw, specifically in *AMY* and *HIP* (**Figure 6**). It is already evident that *SPHK1* plays crucial roles in microglia-dependent neuroinflammatory cascades. Additionally, we also found *PTX3*, which is another marker of microglia involved in regulation of phagocytic activity to be consistently downregulated across DDM of the human brain, specifically in *AMY* and *HIP* (**Figure 6**). Based on these findings, we further tried to elucidate expression of *SPHK1* and *PTX3* in long-term culture of human microglia.

Our results have demonstrated the appearance of 3D cellular aggregates on fifth DIV with differential expression of *CD24* and *CD184* in both flow cytometric and confocal imaging analysis (**Figure 10**). These cellular aggregates (EBs) were further induced to form early microglial precursors as evident by expression of *CD68* and *AIF1/IBA1* on 21DIV as shown in the flow cytometry micrographs (**Figure 10A**). These precursors later gave rise to microglial structures on 34DIV with distinct expression of two mature microglial markers *SPHK1* and *PTX3*, as evident in immunofluorescence-based studies (**Figure 10B**). This study has explored the optimal microenvironment for establishing cellular model mimicking human microglia and the associated important biomarkers.

DISCUSSION

Albeit microglia constitute approximately 10% of the total cell population in the CNS (67), there are major lacunae in the knowledge regarding their expression across human brain ages

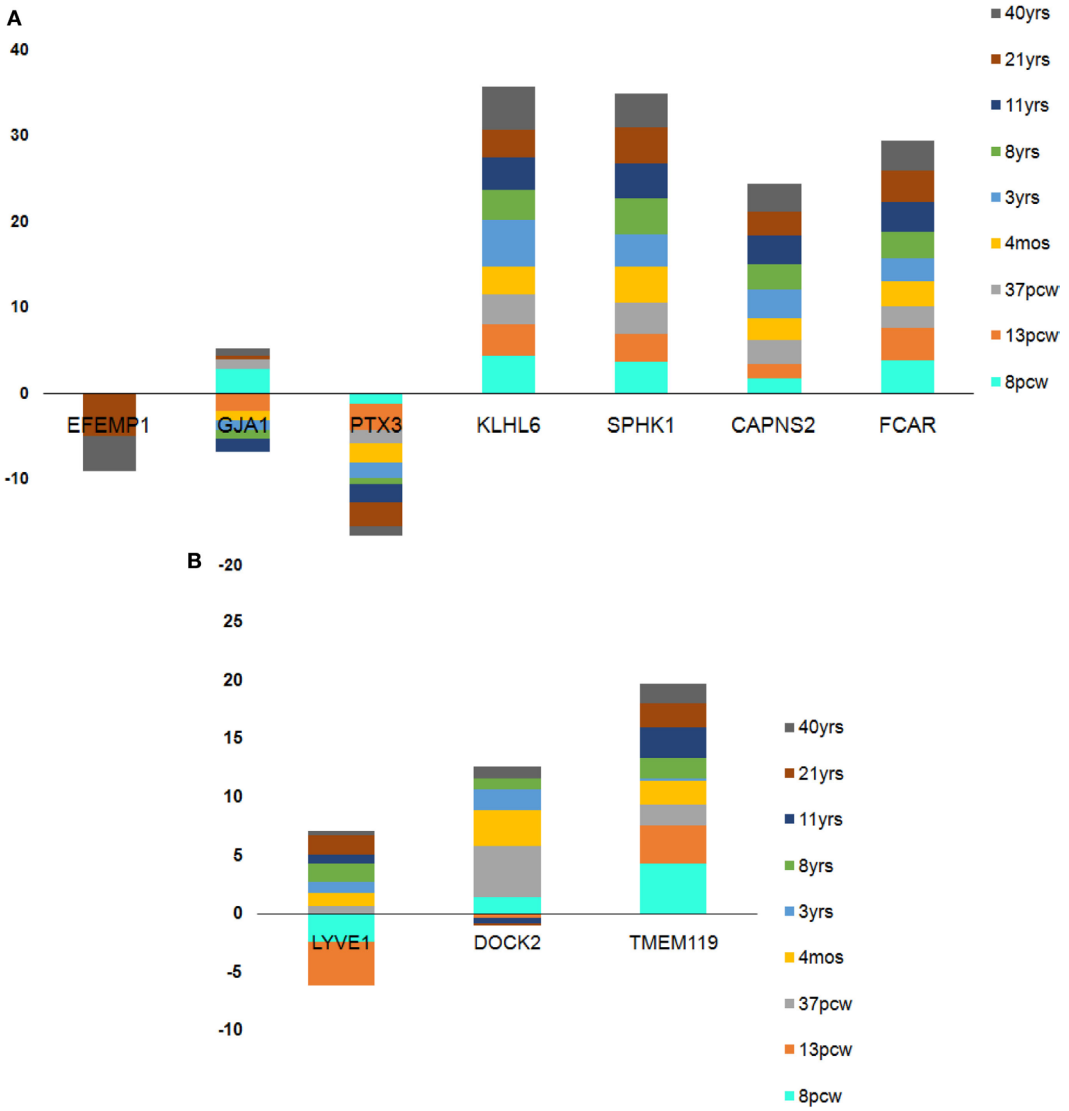

FIGURE 8 | Age-wise dynamics yielded crucial microglia-specific signatures. **(A)** Seven genes found to be consistently high in expression from pre- to prenatal brain developmental stages. **(B)** Three genes found to be dynamically expressed with sharp dips at the transition stage of 37 postconception week (pcw) except in case of *DOCK2* which was inconsistently expressed throughout pre- and postnatal stages. Each stack in the stacked bar plot represents a single brain age whose height denotes the amount of expression.

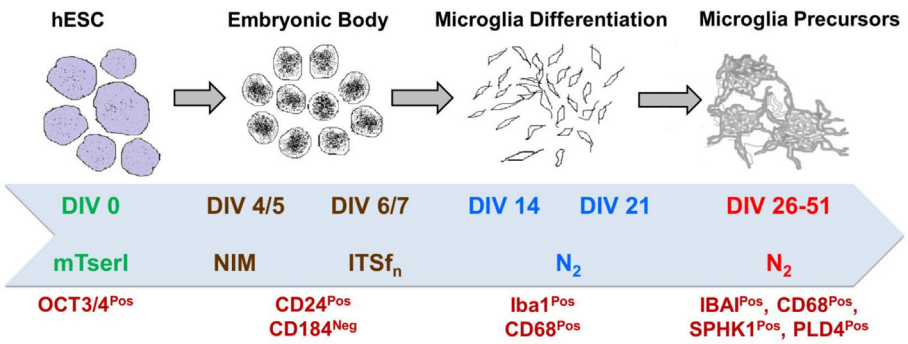

FIGURE 9 | Brief scheme demonstrating the protocol of directed differentiation of hESCs into human microglia which is characterized by several stage-specific markers.

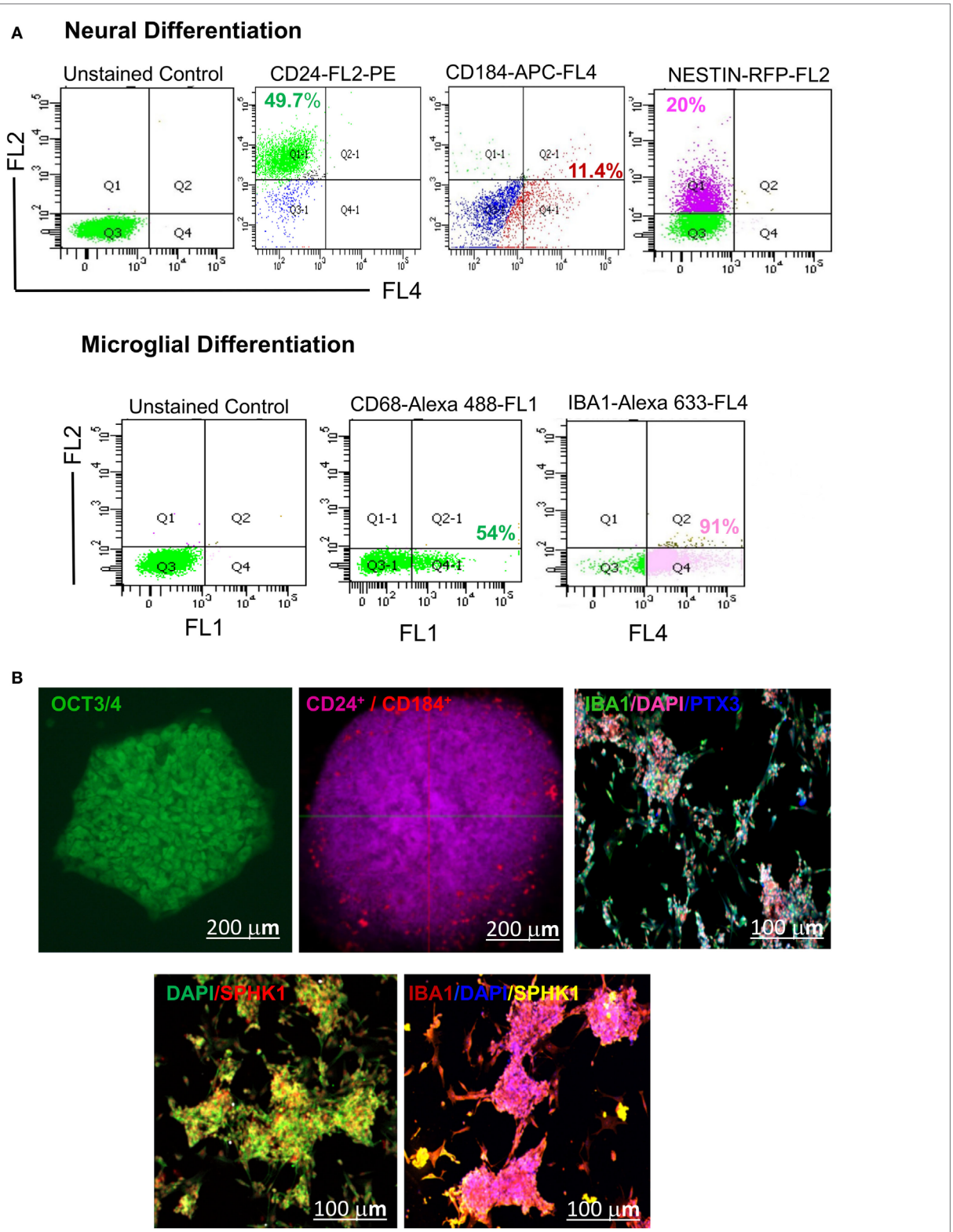

FIGURE 10 | Identification of hESC-derived microglia precursors *in vitro* using different stage-specific markers. **(A)** Flow cytometry based analysis demonstrated characterization of microglia induction and differentiation of microglial precursors. The analysis indicated differentiation of hESCs into embryonic bodies (EB, 5 DIV), as shown by expression of ~49.7% *CD24*^{Pos} and 11.4% *CD184*^{Pos} population. This is followed by neural induction as shown by 20% of *NESTIN*^{Pos} cells. Neurally induced cells further showed the generation of a distinct population having 54% of *CD68*^{Pos} cells and 91% *IBA1*^{Pos} cells. **(B)** Confocal imaging of hESC-derived EBs showed expression of *CD24* and *CD184 in vitro* at 5 DIV. The emergence of mature microglial precursors could be detected on 34DIV, as shown by *IBA1*, *SPHK1*, and *PTX3* expression. DAPI = nuclear marker, DIV = days *in vitro*. The scale bar represents size in micrometers.

and regions (68). Many critical questions involving their ontogeny, localization, and self-renewal still need to be addressed. Discovery of novel microglial biomarkers and elucidation of their specific roles in brain developmental processes will shed light on the onset of neurological disease and progression.

Studies involving human microglia-derived transcriptomics and proteomics are still in its infancy, and given the low scope of availability of healthy/diseased human brain tissue, it is imperative to perform high-throughput data analysis to be able to identify and narrow down crucial microglia signatures (33). In this direction, using available tissue based transcriptomics (69) as well as induced microglia microarray data generated from pluripotent stem cells (25), we analyzed the differentially regulated gene sets pertinent to microglia biology and homeostasis, which resulted in a novel catalog of microglial biomarkers. Further using GSEA on the ascertained list, we have identified different clusters of gene sets specific to human microglia, which revealed several crucial clusters such as metalloproteinases, GPCRs, proteoglycans, immunoeffectors, gap junction family genes, and synaptosomal receptors, etc. These prominent gene clusters explained multidimensional roles and biological activity of microglia. This GSEA map acted as a primer for further downstream analyses. Correlation analysis further explained the differential expression behavior of many genes between the human brain cell types and induced microglia data sets, *viz*., *primary* and *secondary* data sets, respectively. Another interesting inference indicates that there might be different gene subsets required for maintaining microglial homeostasis and activity in the human brain, while enforced microglial differentiation *in vitro* requires an exclusive set of gene regulators. To understand the protein–protein interactions among the shortlisted candidates, we constructed a primary protein neighborhood network and performed pathway analysis. The study highlights some novel pathways associated with microgliasuch as Glycosaminoglycan metabolism, RAP1, Phospholipase C/D, *PPAR*, Fatty acid CoA signaling pathways. Upon assessment of the DDM across brain age groups, we have identified the top 20 altered genes as molecular signatures to developmentally track microglia in fetal, infant, child, adolescent, and adult brains.

Both GSEA and correlation analysis pointed out three possible hypotheses which might play roles in microglia-NPC crosstalk. The first hypothesis suggests that enhanced expression of *PTGS1/2* is linked to *EP2* receptors in microglia and might be the key to crosstalk with the qNSCs that express specifically *PGE2*. The second hypothesis discusses the interaction between microglia and qNSCs through plausible crosstalk between *DOCK2* and S1P, as evidence suggests qNSCs specifically express S1P as biomarker and microglia express SPHK1, *DOCK2*, and *S1PR1/5* (S1P receptor 1/5). The third hypothesis suggests that *GPR183* acts a conditioned rheostat for NSC quiescence and activity in *NOTCH1* dependent pathway. Since differential expression analysis showed high expression of *GPR183* in microglia, and *NOTCH1* regulates self-renewal of hippocampal NSCs, there can be possible molecular crosstalk (70, 71).

Studies from past few decades have indicated both specific and context-dependent localization and activity of microglia during neuroinflammatory circumstances (18, 39). However, hardly any study exists which could track their activity throughout brain development. Another major roadblock that is faced is the unavailability of human brain cells. To address such issues, we established a cellular model of the human microglial cell population and deciphered the expression of existing biomarkers, especially *CD68*, *AIF1 (IBA1)*, *SPHK1*, and *PTX3*. Earlier findings have indicated that SPHK1 is found to be abundantly expressed in several rat brain cell types, including neurons in the hippocampus, cerebellar granule cells, and astrocytes and also in the amoeboid microglial cells of the corpus callosum of the postnatal brain (72). The *in silico* data strongly corroborated with this existing evidence, which suggested that *SPHK1* expression is significantly upregulated in microglia-dense regions, namely hippocampus and amygdala (**Figure 6**). The *in vitro* long-term culture study also supported these findings and suggested that human microglia precursors from post 26DIV showed consistent expression of *SPHK1* in late microglial precursors (**Figure 10B**). The results have also represented another unique marker, *PTX3* in the in cultured human microglial precursor cells, which is known to regulate the phagocytic activity of microglia (44). This observation supported the DDM data which has shown *PTX3* to be consistently downregulated across different ages in HIP and AMY of healthy brains. In summary, our *in vitro* study has validated the emergence of the microglial signatures in hESC-derived human microglial precursors and also represented a robust human microglial model for future investigations involving microglial biology and activity.

Microglia has been associated with onset, aggravation, and progression of neurodegenerative diseases like Alzheimer's, Dementia, and Glioma (73–76). This is primarily due to dysregulated microglial activity during development as well as disease leading to severe neurocognitive impairments and neuronal dysfunction. Overall, this study has unraveled fundamental information underlying the labyrinthine molecular circuitry in microglia functioning, physiology, homeostasis, and genetics across brain developmental stages and regions.

CONCLUSION

Our study has introduced a novel integrative biology approach for tracking human microglia signatures across 25 developmental stages spanning fetal, infant, adolescence, and adult, and delineated their localized expression in microglia-dense regions. The *in vitro* data have also validated the expression of some of the biomarkers in human microglial precursors. Precisely, tracking of microglia transcriptomic dynamics based on brain anatomy and age could establish an essential DDM comprising highly altered gene clusters. This map can be further utilized to develop set of putative prognostic markers in accordance with the age of the human brain. One of the interesting readouts of the DDM was the dynamic expression associated with 37 pcw, i.e., a transition period between prenatal and postnatal brain. To highlight, the study has led to the discovery of microglia age-specific gene signatures, including *EFEMP1*,

GJA1, *PTX3*, *KLHL6*, *SPHK1*, *FCAR*, and *CAPNS2*, which can be further explored for understanding their roles in microglial biology in the human brain.

FUTURE PERSPECTIVES

In essence, this study has established a repertoire of microglia-specific signatures involved in the developing human brain. Characterization of mechanistic roles of these gene fingerprints in experimental models would further bridge the gap in understanding the molecular mechanisms involved in microglia ontogeny, renewal, and maintenance. Age-wise expression profiling of these novel signatures (DDM) in both pre- and postnatal brain stages can act as guidance cues for designing future prognostic and diagnostic biomarkers against neurodegenerative diseases. Finally, the data associated with age and region-specificity can pave the way to personalized medicine interventions for debilitating neurological diseases.

AUTHOR CONTRIBUTIONS

RA: performed analyses, computations, and graphics. RA and SP: designed the methodological approach and wrote the manuscript. SP and SS: read, critically analyzed, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fneur.2018.00266/full#supplementary-material.

FIGURE S1 | Donut plot showing various protein classes associated with the differentially expressed gene set or 3,290 genes.

FIGURE S2 | Correlation matrix/correlogram showing varying correlation ranging from very weak to very strong (0.0–0.99) among significant brain ages within three microglia-enriched regions, namely AMY, HIP, and STR. The color bar depicts the range of correlation coefficient (−1, 1) and in each correlogram, the colored slice in the pie increases in size and intensity in accordance with the correlation coefficient value.

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REVIEW
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Brain-Resident Microglia and Blood-Borne Macrophages Orchestrate Central Nervous System Inflammation in Neurodegenerative Disorders and Brain Cancer

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Inflammation is a hallmark of different central nervous system (CNS) pathologies. It has been linked to neurodegenerative disorders as well as primary and metastatic brain tumors. Microglia, the brain-resident immune cells, are emerging as a central player in regulating key pathways in CNS inflammation. Recent insights into neuroinflammation indicate that blood-borne immune cells represent an additional critical cellular component in mediating CNS inflammation. The lack of experimental systems that allow for discrimination between brain-resident and recruited myeloid cells has previously halted functional analysis of microglia and their blood-borne counterparts in brain malignancies. However, recent conceptual and technological advances, such as the generation of lineage tracing models and the identification of cell type-specific markers provide unprecedented opportunities to study the cellular functions of microglia and macrophages by functional interference. The use of different "omic" strategies as well as imaging techniques has significantly increased our knowledge of disease-associated gene signatures and effector functions under pathological conditions. In this review, recent developments in evaluating functions of brain-resident and recruited myeloid cells in neurodegenerative disorders and brain cancers will be discussed and unique or shared cellular traits of microglia and macrophages in different CNS disorders will be highlighted. Insight from these studies will shape our understanding of disease- and cell-type-specific effector functions of microglia or macrophages and will open new avenues for therapeutic intervention that target aberrant functions of myeloid cells in CNS pathologies.

Keywords: neuroinflammation, tissue-resident macrophages, microglia, neurodegeneration, cancer

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INTRODUCTION

The brain has long been regarded as an immunologically privileged site in which the presence of the blood–brain barrier (BBB) restricts the entry of blood-borne immune and inflammatory cells to the central nervous system (CNS) [for review, see Ref. (1)]. Consequently, key functions in tissue homeostasis and immune defense were attributed to brain-resident cell types, such as microglia or astrocytes (2, 3). Microglia are regarded as the innate immune cell of the CNS. As part of their routine surveillance, microglia continuously monitor their surrounding with

motile protrusions to sense and resolve any disturbance (4). Along with their well-established role as immediate responders to injury and infection (5, 6), there has been an increasing appreciation of the importance of microglia for normal CNS development and function, including developmentally regulated neuronal apoptosis, neurogenesis, myelogenesis, and synaptic pruning (7–9). Given their central role in CNS inflammation, it is not surprising that dysregulation of microglial activation and microglia-induced inflammation is observed in virtually all brain malignancies, including neurodegenerative disorders as well as primary and metastatic brain cancers. Blood-borne immune and inflammatory cells have recently emerged as an important component of the disease-associated microenvironment in the brain and are regarded as critical mediators of progression in neurodegenerative disease and brain cancers. However, the lack of experimental systems that distinguish between recruited and brain-resident myeloid cells has previously halted analysis of cell-type-specific functions in CNS inflammation. The development of new methodologies provides unprecedented opportunities for comprehensive in-depth analyses of the immune landscape of the CNS under steady-state and pathological conditions. Single-cell RNAseq or mass cytometry (CyTOF) allow for an unbiased view on the immune milieu of the brain parenchyma and adjacent boundaries. In addition to the well-characterized macrophage populations of non-parenchymal areas of the brain (10), it is increasingly recognized that various immune cell populations including a large diversity of lymphoid and myeloid subpopulations are present in particular in the meninges and the choroid plexus (11–14). Analysis of parenchymal myeloid cells also revealed high cellular heterogeneity. The existence of distinct myeloid cell phenotypes may reflect functional diversity, different ontological origins, or various cell differentiation states already at steady state (11). The question how environmental cues in different brain malignancies sculpt transcriptional profiles and epigenetic states of microglia and recruited myeloid cell populations during disease progression has recently gained attention. A growing number of studies seek to unravel the heterogeneity of the disease-associated immune landscape to functionally link different cell states to disease progression. Detailed knowledge of the impact of individual cell populations or activation states across different CNS malignancies is critical for the development of improved therapeutic strategies to target dysfunctional cells without affecting essential physiological or beneficial functions. The aim of this review is to discuss recent insights into the cellular and molecular identity of the heterogeneous population of cerebral myeloid cells in different CNS disorders to highlight common and unique features of the distinct subpopulations in the respective CNS pathologies.

ONTOLOGICAL ORIGIN OF MYELOID CELLS IN THE CNS IN HEALTH AND DISEASE

Microglia, the brain-resident macrophages, represent the largest population of myeloid cells in the CNS and are localized in the brain parenchyma. The term microglia was first coined by Pio del Rio-Hortega to describe the non-neural, non-astrocytic "third element of the nervous system" that is distinct from neuroectodermal oligodendroglia and oligodendrocytes. Del Rio-Hortega's findings indicated a mesodermal origin of microglia [for historical review, see Ref. (15)]. However, there was a long-lasting debate on the ontological origin of microglia. An alternative hypothesis proposed that microglia originate from neuro-ectodermal-derived glioblasts (16). This theory was seemingly supported by the findings that donor bone marrow cells failed to contribute to the adult microglia population in either newborn (17) or adult rodents (18). Hickey and Kimura demonstrated that in bone marrow chimera only perivascular microglia derived from the bone marrow (19). The authors used the term perivascular microglia for the cell population that to date is referred to as perivascular macrophages that are located in the Robin-Virchow space. Further evidence that resident microglia are not replaced by cells from the bone marrow was provided by Lassmann et al. (20). The definitive proof for a mesodermal origin of microglia was achieved through a genetic study that showed that mice lacking the crucial transcription factor for myeloid cells, PU.1, are devoid of microglia (21, 22).

Even after the myeloid origin of microglia was proven, debate about the nature of microglia progenitors remained. Controversy was mainly caused by the fact that there are two major sites of hematopoiesis during embryogenesis: the yolk sac and the fetal liver. As depicted in **Figure 1**, primitive hematopoiesis in mice is initiated in the yolk sac at around E7.0, which leads predominantly to the generation of macrophages and erythrocytes (23). Yolk sac-derived primitive macrophages enter the embryo proper after the circulatory system has been established (from E8.5 to E10) (24) and populate various organs that contain tissue-resident macrophage populations, including the brain. Population of the fetal brain by primitive macrophages takes place before the onset of monocyte production by the fetal liver and before the establishment of the BBB. A second wave of "definitive" hematopoiesis is initiated by hematopoietic progenitors that are generated in the yolk sac and the AGM (aorta, gonads, and mesonephros) region of the embryo proper and that migrate into the fetal liver around E10.5. After E11.5, the fetal liver serves as the major hematopoietic organ and generates all hematopoietic linages including monocytes (25). In contrast to primitive hematopoiesis, definitive hematopoiesis depends on the transcription factor Myb (26). Around birth, hematopoiesis starts to be restricted to the bone marrow (27). It further remained elusive if under physiological conditions, monocytes contribute to the establishment of the post-natal and adult microglia population. Fate mapping studies using Runx1MerCreMer lineage tracing model, in which exclusively yolk sac-derived progenitors and their progeny are fluorescently labeled following a tamoxifen pulse at E7.25, have now established that microglia are derived from yolk-sac progenitors that generate a long-living population with self-renewal capacity (28). It was further demonstrated that microglia develop from erythro-myeloid progenitors (EMP) in a stepwise PU.1 and IRF8-dependent manner (29, 30) (**Figure 1**). The development of microglia and primitive yolk sac macrophages is completely dependent on colony-stimulating factor 1 receptor (Csf1r) signaling (28). Microglia are absent in

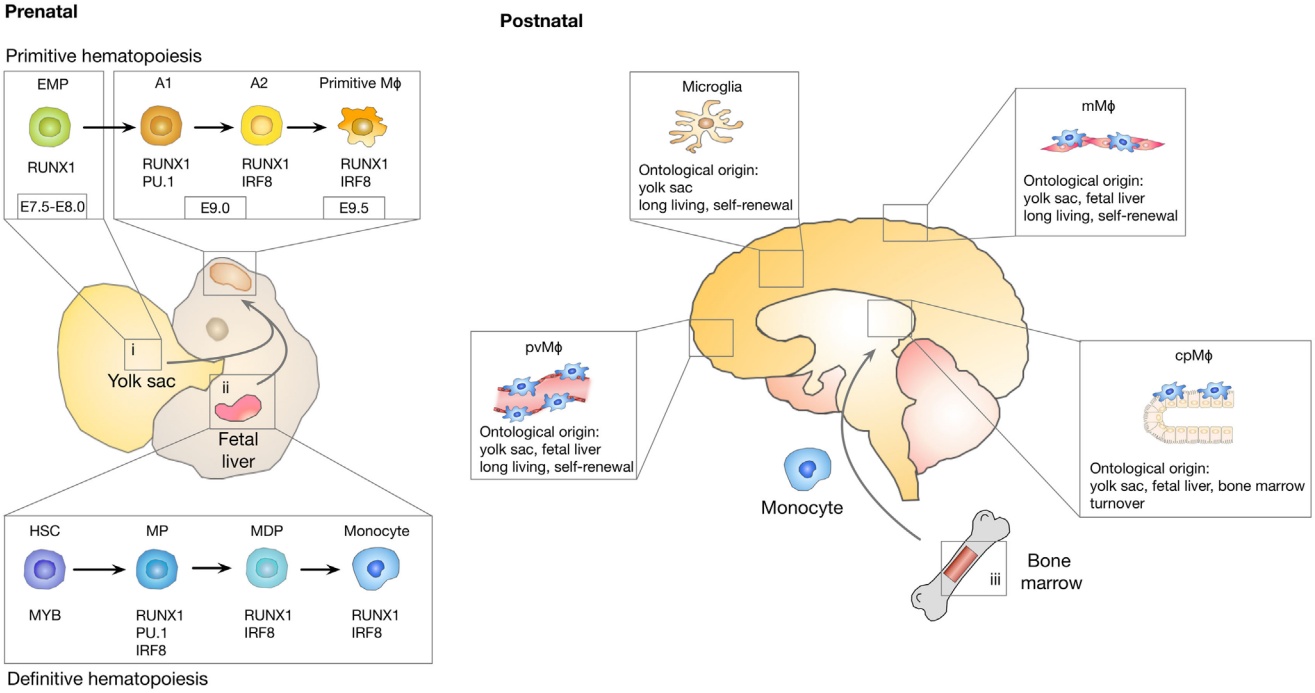

FIGURE 1 | Ontological origin of macrophage subpopulations in the central nervous system (CNS). A first wave of myeloid cell development takes place in the yolk sac (i) between E7.0 and E8.0 in a process known as primitive hematopoiesis that leads to the generation of erythro-myeloid progenitor (EMP) cells. EMP cells give rise to A1 (cKit^{+}Cx3cr1^{-}) cells followed by A2 (Cx3cr1^{+}) cells that differentiate into microglia, perivascular macrophages (pvMϕ), meningeal macrophages (mMϕ), and choroid plexus macrophages (cpMϕ). Microglia originate exclusively from yolk sac-derived progenitors, while non-parenchymal CNS macrophages are replenished with fetal liver-derived progenitor cells (ii) as part of definitive hematopoiesis. Perinatally, hematopoiesis starts to be restricted to the bone marrow (iii). Among the CNS macrophages, cpMϕ are the only population with substantial constitution from bone marrow progenitors. Microglia, pvMϕ, and mMϕ are considered to be long-living cells that regenerate through self-renewal.

Csf1r knock-out mice, while mice lacking functional Csf1 did not show the same severe phenotype (31, 32). This observation was later explained by the existence of a second ligand for Csf1r, namely IL34 (33) that is highly expressed in the brain (34). Microglia represent the only tissue-resident macrophages that are exclusively derived from yolk sac-derived progenitors. By contrast, tissue-resident macrophages in other organs such as Kupffer cells in the liver, alveolar macrophages in the lung, or Langerhans cells in the skin comprise mixed populations and are repopulated by cells originating from the fetal liver during definitive hematopoiesis (27, 35). In light of recent experimental insight, it became apparent that previous findings that indicated a contribution of blood-borne monocytes to the adult microglia pool were confounded by experimental caveats that conditioned the brain for engraftment of peripheral myeloid cells, such as irradiation or parabiosis bias (36). Mildner et al. demonstrated that the use of head shields during myoablative irradiation prior to bone marrow transplantation prevented the recruitment of bone marrow-derived cells into the brain (37). These findings were further supported by studies using parabiosis in mice without the need for irradiation (38). Although chimerism in the periphery reached about 50%, there was no evidence for recruitment of peripheral monocytes to the brain. Moreover, even in the context of inflammation, when monocytes contribute to the inflammatory milieu, blood-borne cells did not integrate into the long-term resident microglia pool (39). The microglia compartment seemed to recover from an internal pool instead. These findings are in line with previous observations demonstrating that peripheral macrophages do not transform and replace microglial cells in EAE models (20). In contrast to these findings, it was shown that under experimental conditions in which the microglial niche is completely vacant in response to microglia depletion strategies, bone marrow-derived cells enter the brain and differentiate into microglia (40, 41). Bruttger et al. recently took advantage of a Cx3cr1CreER-based system (Cx3cr1-iDTR mice) (42) that allows for conditional depletion of microglia without the necessity of generating bone marrow chimera (43). The authors demonstrated that the repopulating microglia arose exclusively from an internal CNS-resident pool. A contribution of bone marrow-derived cells was only observed in mice that were irradiated and additionally received a bone marrow transfer. Moreover, it was demonstrated that microglia self-renewal is dependent on IL1 signaling, while reconstitution from bone marrow precursor is IL1 independent. However, until recently the actual turnover rate of microglia in the brain remained elusive. Employing a multicolor fate-mapping model, the microfetti mouse [a microglia-restricted modification of the confetti mouse (44)], Tay et al. recently analyzed the rate of self-renewal of microglia in steady state, after induced CNS pathology and during the subsequent recovery phase. This study

revealed heterogeneous rates of microglia replenishment in different brain regions (45). Following CNS damage, the authors found a shift from a pattern of random self-renewal within the microglial network toward a rapid expansion of selected microglia clones. This finding provides important insight into the question if microglia are recruited from adjacent regions to sites of CNS damage, or if clonal expansion results in microglial accumulation. Results obtained in the Microfetti mouse clearly favor the latter hypothesis. During the recovery phase in which microgliosis is resolved, the restoration of microglial cell density occurred through egress and apoptotic cell death (45).

Taken together, the field has reached consensus regarding the origin of microglia and the contribution of bone marrow precursors to the microglia pool under steady-state conditions. However, the debate on the functional contribution of yolk sac-derived microglia and blood-borne monocytes in CNS inflammation and their functional interplay is still in its infancy. As discussed in more detail in the following paragraphs, there is evidence that in response to inflammatory conditions associated with, e.g., irradiation, neurodegenerative disorders, or CNS cancer, the recruitment of monocytes or other bone marrow-derived progenitors can supplement the microglial pool. However, it remains unclear if the recruited cells persist and become an integral part of the microglial population, or if those cells represent a transient population that vanishes once the inflammatory stimulus is resolved. Another question that still needs to be addressed in more detail is, if yolk sac-derived microglia and bone marrow-derived macrophages (BMDM) exert redundant or cell type-specific functions in CNS pathologies and if the ontological origin determines responses against therapeutic intervention.

SHAPING OF CELLULAR IDENTITY BY THE TISSUE ENVIRONMENT

To understand the imprinting of disease-associated states on microglia and monocyte-derived macrophage identity in more detail, it is important to first consider the effects of specialized tissue environments on tissue-resident macrophages. It is increasingly recognized that in addition to the ontological origin, environmental factors play a critical role in defining functionality of tissue-resident macrophages and determine the fate and persistence of cells in tissues. Consistent with their diverse locations and functions, tissue-resident macrophages in different organs display distinct gene expression profiles (46, 47). Several studies have already dissected the genetic and epigenetic imprinting of specific tissue-resident macrophages and identified a range of transcription factors that are essential for cell type restricted gene expression profiles, e.g., SpiC for red pulp macrophages (48, 49) and GATA6 for peritoneal macrophages (46, 50). Two recent studies undertook the effort to systematically characterize the genetic and epigenetic imprinting of tissue-resident macrophages in specific organ environments. Both studies used RNA sequencing in combination with chromatin immunoprecipitation (Chip)-Seq (51) and assay for transposase-accessible chromatin (ATAC)-Seq (52) to identify enhancer regions that are coupled to gene expression and accessible chromatin (53, 54). The studies by Lavin and Gosselin indicate that tissue-resident macrophages share epigenetic structures and gene expression with other myeloid cell populations. Similarities within the lineage are largely determined by collaborating transcription factors (CTFs) such as PU.1 and lineage-determining transcription factors, including interferon regulatory factor family members and CCAAT/Enhancer-Binding-Protein (Cebp)-a (55–57). However, each tissue additionally has its unique gene expression profile that is controlled by changes in enhancer landscapes in response to environment-specific signals (**Figure 2**). Interestingly, both studies describe pronounced differences in enhancer landscapes among macrophage subtypes, while promoters are largely shared across different macrophage subpopulations and even between macrophages, monocytes, and neutrophils. It was demonstrated that microglia are most distinct from other tissue-resident macrophages in terms of their genetic landscape (53). This comparison also revealed that macrophage populations that are exposed to similar environmental cues converged to similar expression patterns. For example, Kupffer cells and splenic macrophages were shown to share a cluster of highly expressed genes that are enriched for gene ontology (GO) annotations, such as heme and porphyrin metabolism, indicating their role in erythrocyte turnover (48, 49). Similarly, small and large intestinal macrophages were shown to express genes enriched for GO annotations that reflect exposure to microbiota, such as response to bacteria and antigen processing. A more detailed comparison between microglia and peritoneal macrophages identified tissue-specific signals that determine the epigenetic and genetic imprinting of microglia and peritoneal macrophages. The genetic landscape of microglia is known to be strongly driven by the presence of TGFβ and IL34 (58, 59), while retinoic acid is a well-characterized environmental factor that dictates genetic imprinting of peritoneal macrophages and is essential for their development and function *via* GATA6 activation (60). The extent of tissue-specific cues on enhancer landscapes was further proven by transplantation experiments in which peritoneal macrophages were transferred to the lungs. Interestingly, the transferred tissue-resident macrophages lost most of their original tissue marks and acquired a tissue program based on their new host tissue (54).

In summary, identification of enhancer landscapes that are imprinted by specific tissue environments together with the notion that environmental cues can override ontological imprinting ultimately leads to the question, how blood-borne monocytes and macrophages are affected by the host tissue upon recruitment to sites of injury, inflammation, neurodegeneration, and neoplastic transformation and also, to which extent, the disease status dominates the imprinting of resident and recruited cell populations. The next paragraph will discuss recent findings from the field of neurodegenerative disorders, with a focus on Alzheimer's disease (AD), and brain cancers that provide critical insight into the heterogeneity of disease-associated myeloid cells.

MOLECULAR IDENTITIES OF MICROGLIA AND MACROPHAGES IN BRAIN MALIGNANCY

The local tissue environment has been shown to sculpt macrophage transcriptional profiles and epigenetic states under

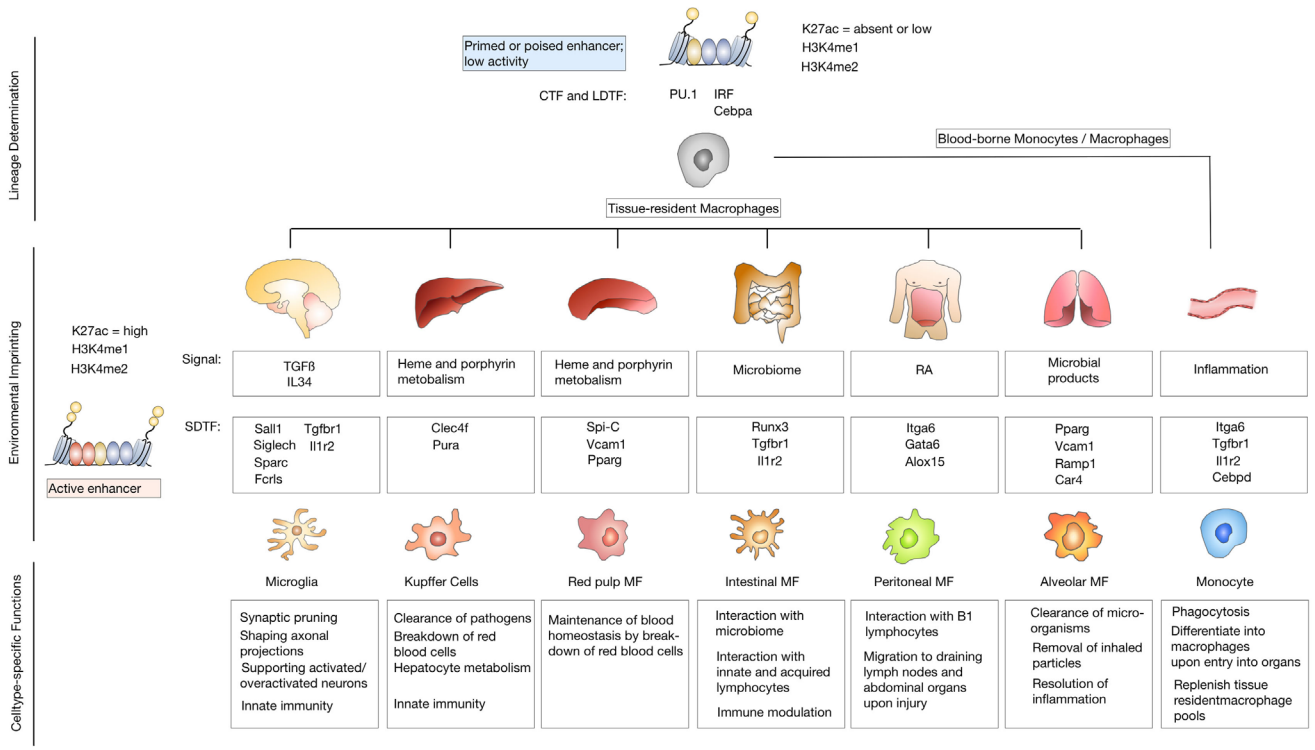

FIGURE 2 | Environmental imprinting of tissue-resident macrophages. Differentiation of precursor cells into specific lineages is determined by binding of lineage-determining transcription factors (LDTFs) and collaborating transcription factors (CTFs) to closely spaced recognition patterns on the DNA. Binding of LDTFs and CTFs selects enhancers as primed or poised. Primed enhancers are marked with characteristic histone modifications such as histone lysine 4 monomethylation (H3K4me1) or dimethylation (H3K4me2). Poised enhancers are defined by the presence of histone H3 lysine 27 trimethylation (H3K27me3). Primed or enhancers show low activity due to the lack of enhancer RNA production or the presence of H3K27me3, that has to be removed to induce an active enhancer state (upper panel). Tissue-resident macrophage populations are exposed to unique environmental cues that lead to genetic and epigenetic imprinting based on signal-dependent transcription factors (SDTF) that bind and activate primed or poised enhancers. Active enhancers are marked by H3K4m1 or H3K4me2 and histone H3 lysine 27 acetylation (H3K27ac) (middle panel). Environmental imprinting induces cell-type-specific functions of different tissue-resident macrophage populations (lower panel).

steady state conditions. However, it remained unclear whether an inflammatory tissue environment may affect differently macrophage populations of distinct ontogenies. To answer this question, it is first essential to determine the extent of peripheral recruitment of myeloid cells to the CNS under distinct pathological conditions. A critical contribution of recruited myeloid cell populations has been proposed for a long time (61). However, given the number of experimental caveats including the requirement of radiation for bone marrow transplantation (36, 37) as well as the route for tumor cell implantation, which often relies on intracranial injection, it remained unclear if the observed infiltration is due to experimental manipulation or represents an integral part of disease progression. Another important aspect that has to be taken into account is species differences that impact the extent of infiltration of cells from the periphery. It was shown that rat models for ischemia or brain cancer show lower rates of infiltration of blood-borne cells than observed in mouse models (62, 63). There is also evidence that recruitment of blood-borne inflammatory cells in human brain cancers is less pronounced than in mouse models (64). New approaches that employ lineage tracing models or single-cell RNAseq provide an unbiased view on the extent of infiltration from the periphery and the disease-associated imprinting on different myeloid subpopulations. Moreover, a growing number of studies addresses these questions on patient-derived samples thereby excluding the possible effects of experimental artifacts and provide important insight into the clinical relevance of experimental data (65–68).

Neurodegenerative Disorders

Neurodegenerative disorders share common features including neuronal loss that ultimately leads to cognitive decline and motor dysfunction, which is associated with the establishment of an inflammatory environment. The immune milieu is comprised predominately of brain-resident microglia, which is supplemented by infiltrating immune cells. While AD, Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS), and multiple sclerosis (MS) are considered as neurodegenerative diseases, it is important to appreciate differences in the extent of peripheral involvement, a critical parameter to define neuroinflammation (69). ALS and MS are autoimmune inflammatory disorders of the CNS that lead to irreversible axonal damage and progressive neurological disability (70, 71). In case of ALS and MS, immune

cell infiltration is causative. For example, in MS, acute demyelinating white matter lesions show myelin breakdown accompanied by infiltration of innate immune cells (i.e., monocytes) and adaptive immune cells (T- and B-lymphocytes) (72, 73). By contrast, symptoms of AD and PD should rather be regarded as innate immune reactions (74). Infiltration of lymphocytes only occurs at late disease stages when integrity of the BBB is lost. The critical contribution of CNS inflammation to disease progression in neurodegenerative disorders has been appreciated since the first description of the pathological parameters in particular due to the manifestation of microgliosis (15, 75). However, to date, it still remains controversial if CNS inflammation, in particular under conditions that trigger pronounced recruitment of myeloid cells from the periphery, is associated rather with disease amelioration or acceleration.

Alzheimer's disease represents the most common form of dementia and AD pathology is characterized by extracellular deposition of amyloid-β peptides that leads to β-amyloid plaques, formation of neurofibrillary tangles composed of hyperphosphorylated tau protein, neuroinflammation, and neuronal loss (76). An accumulating number of studies seek to evaluate the functional role of brain-resident microglia and to dissect the contribution of recruited myeloid and lymphoid cells. While some studies propose a protective role of microglia in AD (77–79), other reports show that under disease conditions, microglia acquire pro-inflammatory properties that have been associated with disease acceleration (80–83). These conflicting data can at least in part be attributed to a high phenotypic and functional heterogeneity of the disease-associated myeloid cell population. For example, Mildner et al. reported distinct and non-redundant roles of microglia and other brain-associated myeloid cells in AD mouse models. This study revealed a dominant role of CCR2-expressing myeloid cells in β-amyloid clearance (84). Previous studies that aimed at dissecting the functional contribution of myeloid subpopulations in disease progression relied on analyses of bulk cell populations categorized by a limited number of markers and single time points for analyses often at end stage disease. Importantly, analysis of bulk populations limits the capacity in resolving the heterogeneity and complexity of the immune milieu within the CNS (85–87). To date, single-cell transcriptomics allows for an unbiased characterization of immune cell types and states, thus systematically resolving the complex heterogeneity of the disease-associated immune landscape in comparison to normal tissue or in response to therapy (30, 88–91). Two recent studies used single-cell RNA seq analysis to characterize the immune landscape at different stages of disease progression in AD using the 5XFAD model (92) or the CKp25 model (93). Using the CKp25 AD-like mouse model, Mathys et al. identified early- and late-response states that differ significantly from homeostatic microglia. The early-response microglia show enrichment of cell cycle genes and genes involved in DNA replication and repair indicating that microglia expansion occurs at early disease stages. At late stages of disease progression, immune-related pathways were dominant and an enrichment of interferon-related response genes was detected. Interestingly, the late stage clusters comprised heterogeneous populations. Based on gene expression signatures of different clusters identified at late stages, the authors conclude that those subpopulations could reflect exposure to type 1 or type 2 interferon, respectively (93). Using the 5XFAD model, Keren-Shaul et al. also identified two distinct microglia states [cluster II and III, referred to as disease-associated microglia (DAM)] in AD that were absent in normal brain. Compared to normal microglia, DAMs showed reduced expression of microglia core genes including the purinergic receptors P2ry12, P2ry13, Cx3cr1, and Tmem119 (58, 94). Concomitantly, DAMs showed enrichment of genes that are known as common risk factors for AD, including Apoe, Ctsd, Lpl, Tyrobp and Trem2. Gene set enrichment analysis indicated induction of lysosomal/phagocytic pathways, endocytosis and regulation of immune response. Interestingly, temporal resolution of the DAM phenotype manifestation indicated a two-step process. The first step appears to be accompanied by suppression of key regulators of microglial phenotype and function, such as Cx3cr1. The second stage was shown to be dependent on Trem2 and Tyrobp/Dap12. Analysis of the spatial localization of DAMs revealed close association to amyloid plaques. Given the enrichment of phagocytic and lipid metabolism pathways, the authors propose that DAM are involved in plaque clearance. The presence of DAM-like cells in AD patients has been demonstrated by histology as well as transcriptomic analysis (66, 92).

In addition to the investigation on DAMs in AD, Keren-Shaul et al. interrogated if DAMs are also present in other neurodegenerative pathologies, including a mouse model for ALS and aging. Interestingly, distinct myeloid subpopulations that showed similarities to DAMs in AD were observed also in response to aging and in ALS (92). Given the finding that progressive neurodegeneration leads to the induction of similar gene signatures in DAMs it is very interesting to compare the results by Keren-Shaul and Mathys. Both studies describe the occurrence of two distinct microglia subpopulations during disease progression that are distinct from the microglia state in the healthy brain. However, early and late disease-stage associated populations are not completely unrelated. Earlier stages rather represent a transient intermediate activation state as part of the reprogramming of homeostatic microglia in response to neurodegeneration. Interestingly, late-response microglia express increased levels of many genes that were also observed to be upregulated in DAM, suggesting a substantial similarity between the expression profiles of DAM and late-response microglia (92, 93). This observation is consistent with the idea that the DAM program may be a primed set of genes that is expressed in response to varied conditions of altered homeostasis. This is further supported by gene expression similarities between AD, ALS and aging. However, the identified populations also show important differences. For instance, Mathys et al. observed that many antiviral and interferon response genes were significantly upregulated in late-response microglia but not in DAM. Moreover, significant differences in the expression of both stage 1 and stage 2 DAM enriched genes were observed in late-response microglia. This was less pronounced in early-response microglia. Differences in gene signatures can be in part be explained by model-specific characteristics. However, this observation might also indicate that the distinct microglia states represent intermediate stages

on a continuum of microglia reprogramming that ultimately converts protective/beneficial functions into neurotoxic functions (92, 93). A comparison of the observed signatures and phenotypes suggests that the early- and late-stage microglia represent the most naïve and most advanced population, respectively, while the DAM stage 1 and stage 2 might occur along the transition from early to late stage microglia. Mrdjen et al. took a similar approach as recently employed by Korin et al. for steady state conditions (11) to investigate the immune landscape in CNS inflammation using mass cytometry (95). The combination of CyTOF together with lineage tracing allowed the authors to identify different subsets of myeloid cells and the phenotypic changes in CNS immune cells during aging, AD and MS with definitive proof of the ontological origin. Microglia phenotypes observed by Mrdjen in an EAE model reflected an inflammatory phenotype that showed similarities to the phenotypes observed in aging and AD mouse models. This indicates a potential universal disease-associated microglial signature as recently proposed (96).

Although the studies by Keren-Shaul and Mathys both provide important insight into the molecular basis of DAMs or early- and late stage microglia, the beneficial or detrimental role of the respective subpopulations remains to be studied in more detail. Keren-Shaul et al. propose a protective function of DAMs due to their contribution in plaque clearance (92). Tyrobp and Trem2 are known to form a signaling complex associated with phagocytosis (97). Trem2 expression is known to be critical for clearance of neuronal debris and loss of function of Trem2 or Tyrbp (Dap12) are associated with dementias characteristic of neocortical degeneration observed in AD. In line with this interpretation, it has previously been reported that Trem2 is critical for microglia clustering and expansion around amyloid plaques and that the Trem2-mediated early microglial response limits diffusion and toxicity of amyloid plaques (79, 82). By contrast, Jay et al. demonstrated that Trem2 deficiency resulted in reduced infiltration of inflammatory myeloid cells and thereby ameliorated AD pathology at early stages (77) and exacerbated it at later stage (98). Hence, one possible explanation might be that DAM function has a transient beneficial impact during the initial phase of AD onset while later stages might be associated with rather detrimental effects. In light of recent insight on microglial stages in AD, Hansen et al. proposed a dichotomous role of microglia, with the detrimental microglia population occurring later in disease course at the time when synapse loss is observed and symptoms manifest (99). As depicted in **Figure 3**, microglia in steady state are protective and AD is prevented by constant scavenging of aβ peptides. Once the equilibrium is lost and Aβ levels accumulate, microglia phagocytose and clear Aβ aggregates. These protective activities involve activation of microglia to a DAM state in a Trem2-dependent manner. Genetic susceptibility or aging can lead to impaired microglia function. Accumulation of toxic amyloid plaques leads to tau pathology in stressed or damaged

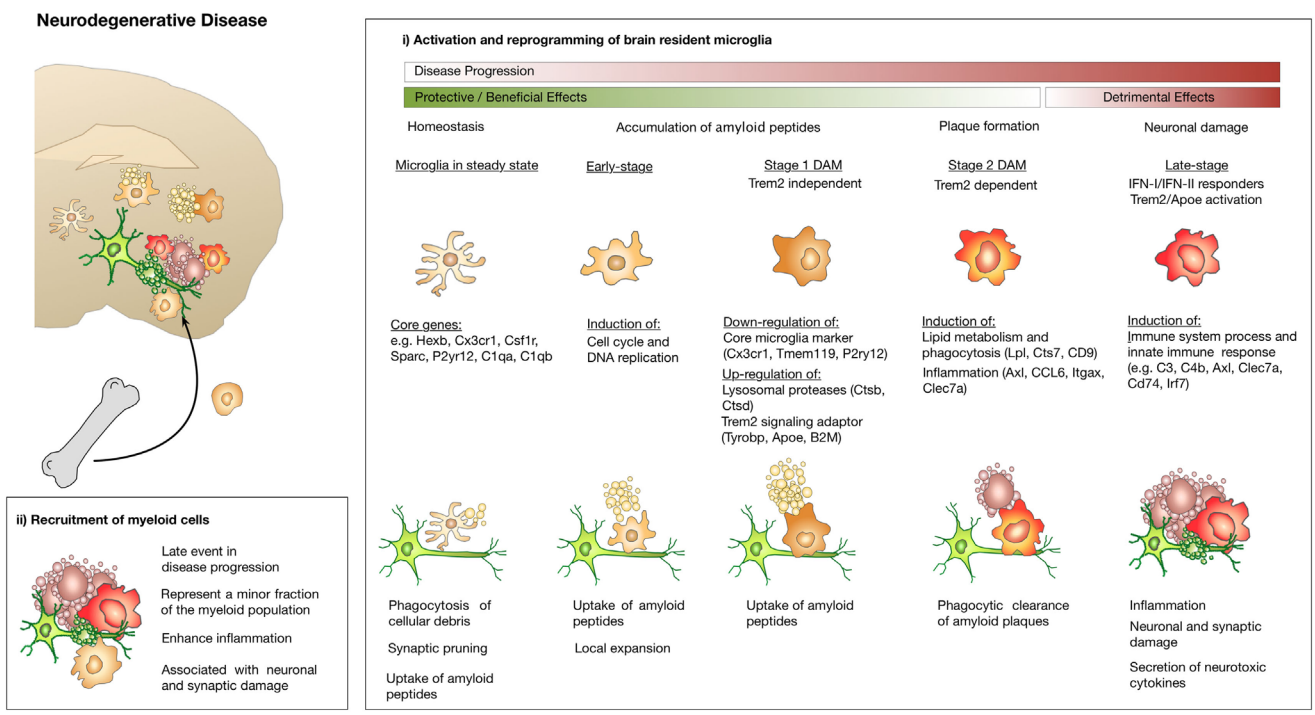

FIGURE 3 | Microglia and macrophage activation states and effector functions at different stages of disease progression in neurodegenerative disease. (i) Microglia exert protective functions including phagocytosis of cellular debris, uptake of Aβ peptides, and clearance of amyloid plaques at early disease stages. Disease progression leads to changes in microglia functions that limit their ability to confine disease manifestation or even induces inflammatory activation states that cause neuronal and synaptic damage. Induction of Trem2/Apoe signaling was shown to mediate conversion of protective microglia into tissue damaging ones. Recruitment of macrophages from the periphery appears to occur at late disease stages and contributes to disease acceleration due to enhanced inflammation.

neurons, which induces an unconstructive and inflammatory state in microglia that causes deleterious neuronal damage (99) (**Figure 3**). This hypothesis is further supported by a recent study using the APP-PS1 model for AD, the ALS model SOD1G93A and an EAE model to investigate the role of the Trem2–Apoe complex in microglial dysfunction in neurodegeneration (96). In contrast to previous studies, Krasemann et al. proposed a neurodegenerative role of microglia in which the Trem2–Apoe pathway regulates the phenotypic switch from neuroprotective to neurodegenerative microglia. A negative feedback loop of Apoe to TGFβ suppressed homeostatic microglia concomitant with an induction of Bhlhe40 response genes including Clec7a, Lgals3, Gpnmb, Itgax, Spp1, Cl2, and Fabp5. Those recent data are in line with previous findings on the presence of functionally distinct or opposing microglia/monocytes populations in EAE models (83, 100). It was demonstrated that monocyte-derived macrophages initiate demyelination, while microglia rather clear debris. Gene expression analysis confirmed that macrophages are highly phagocytic and inflammatory, while microglia showed globally suppressed metabolism at early disease stages (83). Gao et al. proposed a dichotomy of the function for Tnfr2 in myeloid cells. It was shown that microglia-derived Tnfr2 signaling is associated with protective effects, while monocytic/macrophagic Tnfr2 stimulated immune activation and EAE initiation (100). Moreover, as discussed in the following paragraph on brain cancers, reprogramming of pro-inflammatory macrophages with anti-tumor functions into immune-suppressive, tumor promoting macrophages has been described for a comprehensive number of cancer entities which is often regarded as de-regulated wound healing programs.

Primary and Secondary Brain Cancer

Massive infiltration of macrophages into tumors has been reported for a large proportion of primary tumor entities and metastasis. Macrophages represent the most abundant stromal cell type in many cancers and comprise up to 30–50% of the tumor mass including CNS cancers such as GBM (101) and brain metastases (102). Functional analyses indicate that the presence of tumor-associated macrophages (TAMs) fosters tumor growth, regulates metastasis and affects therapeutic response (103–105). Accumulation of TAMs is often associated with poor patient prognosis (106, 107).

To date only few studies have looked at the origin and fate of macrophages during cancer progression at the primary site and in metastasis. One of the first reports that systematically dissected the cellular and molecular origin of tumor-associated macrophages employed parabiosis experiments in the PyMT mouse model for breast cancer (108). The authors demonstrated that tumor development triggers a unique innate immune response that is characterized by the differentiation of inflammatory monocytes into tumor-associated macrophages (TAMs). Terminal differentiation of monocytes into TAMs occurred in a notch-dependent manner *via* the recombination signal binding protein for immunoglobulin regulator (Rbpj) (109). Monocyte-derived TAMs showed pronounced transcriptional differences compared to resident mammary-tissue-macrophages (MTM). TAM expansion during breast cancer progression led to a loss of MTMs (108). By contrast, it was recently demonstrated in PDAC models for pancreatic cancer that tissue-resident macrophages persist and undergo significant expansion. TAMs in PDAC tissues adopted a transcriptional program that is associated with cell proliferation (110). This effect was shown to be enhanced by tissue-resident macrophages derived from the yolk sac or fetal liver, but not by HSC-derived monocytes/macrophages. Consequently, macrophages of different ontological origins had different impact on tumor progression in the PDAC model. Loss of monocyte-derived macrophages only showed marginal effects on tumor progression, while depletion of tissue-resident macrophages significantly reduced tumor progression (110). The ontological origin of TAMs in primary brain cancer was investigated in several recent studies using different glioblastoma (GBM) mouse models (101, 111–113). The study by Muller et al. used bone marrow transplantation strategies with head protected irradiation (HPI) in the GL261 model in direct comparison to total body irradiation (TBI) (113). Avoiding previously reported irradiation bias in the head region, the authors demonstrate that recruited macrophages contribute only at later stages to the tumor mass and constitute around 25% of the myeloid population. Interestingly, tumor progression in TBI-treated mice was accelerated compared to the HPI cohort, suggesting that recruited macrophages contribute in promoting tumor growth, yet their infiltration might be predominately caused by impact of IR (113). In order to fully circumvent the necessity of IR, two recent studies employed a genetically engineered model for proneural GBM and the GL261 model in combination with different lineage tracing models to discriminate ontologically distinct subpopulations (111, 112). Using the $Cx3cr1^{GFP/wt}$:$Ccr2^{RFP/wt}$ double knock-in model (114, 115) (see also **Table 1**), Chen et al. demonstrated that $Cx3cr1^{lo}Ccr2^{hi}$ monocytes are recruited to GBM, where they differentiate into $Cx3cr1^{hi}Ccr2^{lo}$ macrophages and $Cx3cr1^{hi}Ccr2^{neg}$ microglia-like cells. In contrast to the results by Muller et al., recruitment of bone marrow-derived monocytes/macrophages was reported to occur at early stages of GBM initiation. Recruited macrophages were predominantly localized to perivascular areas, while microglia were found in peri-tumoral regions. Quantification of the extent of infiltration suggested that recruited macrophages constitute up to 85% of the TAM population, with the remaining 15% being represented by microglia. A possible explanation for the discrepancy of the observed influx in both studies might be differences in tissue harvest strategies. Chen et al. focused their analysis on macro-dissected tumor areas while Muller et al. processed the entire tumor-bearing hemisphere with considerable involvement of adjacent non-tumor-bearing brain parenchyma. Moreover, it is important to note that the study by Muller et al. provided evidence that GBM-associated microglia upregulate CD45 and represent an inherent part of the $CD45^{hi}$ population in the tumor context. Upregulation of CD45 expression on microglia was previously demonstrated in response to different inflammatory stimuli (116) and underlines the fact that CD45 levels as proposed by Ford et al. (117) can only be used to discriminate macrophages and microglia under steady-state conditions while it is not suitable under inflammatory conditions. It was also demonstrated that activated microglia including GBM-associated microglia downregulate Cx3cr1 (112).

TABLE 1 | Lineage tracing models and marker to distinguish microglia and monocyte-derived macrophages in the brain.

Approach	Cell type specificity	Principle	Advantages	Limitations	Reference
(a) Transplantation models					
BMT; TBI	BMDM	HSC source of blood monocytes is replaced with modified/labeled HSCs	High chimerism No time consuming crossing into genetic disease models	Variability in myeloablation and reconstitution Artificial engraftment of BM cells in the CNS	(113)
BMT; HPI	BMDM	HSC source of blood monocytes is replaced with modified/labeled HSCs	High chimerism No time consuming crossing into genetic disease models	Variability in myelo-ablation and reconstitution	(37, 113)
BMT; Busulfan	BMDM	HSC source of blood monocytes is replaced with modified/labeled HSCs	High chimerism No time consuming crossing into genetic disease models Chemical myeloablation No irradiation	Variability in myeloablation and reconstitution	(112)
Parabiosis	BMDM	HSC source of blood monocytes is replaced with modified/labeled HSCs	Constant influx of donor cells No myeloablation required No time consuming crossing into genetic disease models	Technically challenging Low chimerism	(39)
(b) Genetic lineage tracing models					
$Ccr2^{RFP/wt}$; $Cx3cr1^{GFP/wt}$	Monocytes (red) MG (green)	Differential labeling of $Cx3cr1^{hi}$: $Ccr2^{neg}$ MG (green) and of $Cx3cr1^{lo}$:$Ccr2^{hi}$ monocytes (red)	MG and monocytes contain reporter for labeling	Recruitment to the brain leads to increased Cx3cr1 levels in monocyte-derived macrophages Ccr2 expression is downregulated in monocyte-derived macrophage upon differentiation	(114, 115)
Flt3-Cre	Monocytes and HSC-derived monocyte precursors	Label/modification induced in $Flt3^{+}$ monocyte precursors	Useful for lineage tracing of myeloid precursors Useful complementary approach to MG restricted lineage tracing	Cre expression or transmittance restricted to male mice	(118)
$Cx3cr1\text{-}Cre^{ER}$	MG	Recombination is induced in all $Cx3cr1^{+}$ cells upon tamoxifen pulse. Long-living MG retain the label/modification. While monocytes vanish and are replenished from precursors that were generated after Cre recombination in response to the tamoxifen pulse	Long-term labeling/modification is restricted to MG	Spontaneous modification reported in one model Low recombination in mMF (40–50%)	(42)
$Sall1\text{-}Cre^{ER}$	MG	Label/modification induced in $Sall1^{+}$ MG	Sall1 expression is stable also in response to different stimuli	Targeting of non-hematopoietic cells in the liver, heart and kidney	(119)
(c) Cell-type restricted marker expression					
CD45	MG^{lo} $BMDM^{hi}$	MG display lower surface expression	No requirement for combination of several markers	Activated in MG upregulate CD45 BMDM in brain malignancies downregulate CD45	(112, 113, 120)
Tmem119 P2ry12 Siglech	MG^{hi} $BMDM^{lo}$	MG show high expression	Applicable for mouse and human	Downregulation in MG during activation BMDM in GBM show increased expression	(58, 94, 112)
Sall1	MG^{hi} $BMDM^{lo}$	MG show high expression	Applicable for mouse and human Stable expression level at different activation levels	Low expression found on non-leukocytes in the liver, heart, and kidney	(119)
Itga4/Cd49d	MG^{lo} $BMDM^{hi}$	BMDM show high expression	Applicable for mouse and human Stable expression level at different activation levels	Expression found on T cells	(112)

BMT, bone marrow transplantation; TBI, total body irradiation; HPI, head protected irradiation; BMDM, bone marrow-derived macrophage; HSC, hematopoietic stem cell; MG, microglia; BM, bone marrow; CNS, central nervous system.

The gating strategy employed by Chen et al. relied on the discrimination of macrophages and microglia by CD45 expression levels and used a mouse model that is based on Cx3cr1 and Ccr2 promoter activity for reporter gene expression (111). Hence, it is possible that the $CD45^{hi}Cx3cr1^{med}$ population represents a mixed population of microglia that upregulate CD45 concomitant with Cx3cr1 downregulation.

Bowman et al. used a comprehensive set of different lineage tracing models to unravel the extent of macrophage recruitment to GBM and brain metastasis (112). The lineage tracing models used in this study were based on specific labeling of microglia or BMDM using the tamoxifen inducible Cx3cr1CreER-IRIS-YFP;R26-LSL-TdTom model (42) or the Flt3Cre;R26mTmG model (118) (see also **Table 1**). Using these complementary lineage-tracing strategies, it was demonstrated that BMDMs contribute to the TAM pool in different GBM and brain metastasis models in the absence of IR. BMDMs constituted approximately 50% of the TAM population in GBM and 25% in brain metastasis. RNA sequencing of FACS sorted myeloid populations revealed distinct clustering of all TAM populations from normal microglia and monocytes. Within the TAM cluster, further cell and tumor type-specific clusters were identified (112). The TAM cluster showed enrichment of cell cycle related genes, upregulation of complement-related factors, extracellular matrix components, proteases, lipid metabolism mediators, and clotting factors. Interestingly, the authors found several microglia-enriched genes (e.g., Tmem119, Olfml3, Lag3, Jam2, and Sparc) to be upregulated in TAM-BMDM, while other microglia genes (e.g., Sall1, P2yr12, and Mef2c) were not induced in TAM-BMDM (112). These results further indicate that macrophages acquire tissue-resident gene expression upon infiltration into foreign tissue as previously proposed by Lavin et al. (54) and Gosselin et al. (53). Analysis of the epigenetic imprinting of TAM-MG and TAM-BMDM revealed enrichment of Fos/Jun and Pu.1 binding sites in both populations. In addition to those shared motifs, it was demonstrated that TAM-BMDM showed enriched enhancer usage for Runx and Creb/bZip motifs, while TAM-MG peaks were enriched for Smad3 and Mef2a. Interestingly, based on enriched genes it appears that TAM-MG rather exert pro-inflammatory functions as evident by an upregulation of cytokines such as Tnf and Ccl4 as well as classical complement components (e.g., C4b, C2, and Cfh), a pathway that was previously shown to be associated with synaptic pruning and host defense (121). By contrast, TAM-BMDM showed gene signatures that indicate functions in wound healing, antigen presentation and immune suppression (112) (**Figure 4**). Insight into the genetic and epigenetic landscape of TAM-MG and TAM-BMDM in GBM provides evidence for complex networks of tissue and disease imprinting that at least in part can be attributed to the ontological origin of the cells and can be linked to functional differences. In the future, it will be very interesting to analyze in more detail the heterogeneity of different subpopulations at different stages of disease progression as recently done for AD mouse models and to perform functional validation of the proposed mechanisms for each subpopulation. Moreover, it is still unknown if brain tumors of different origin such as oligodendroglioma or brain metastasis induce similar genetic and epigenetic imprinting like gliomas or if the signatures are fundamentally different.

In addition to the important insight into the identity of TAM populations in GBM, Bowman et al. took advantage of the RNAseq data obtained from lineage tracing models to identify markers that discriminate macrophages and microglia in a tissue- and disease independent manner. The authors identified and validated Itga4/CD49d that is specifically repressed in microglia, as a marker to distinguish BMDMs from microglia in primary and metastatic brain cancer in mouse models as well as human samples (112). A list of lineage tracing models and markers that allow for discrimination of microglia and recruited myeloid cells and highlights advantages and limitations is provided in **Table 1**. For review see also (122).

Within the last few years, a growing number of studies performed in-depth analyses on GBM patient samples. While the primary focus of these studies was rather on tumor cell centric questions, the results obtained from GBM sequencing allow conclusions on the tumor microenvironment. Wang et al. reported increased infiltration of macrophages during disease progression with the highest extent of macrophage/microglia accumulation in mesenchymal GBM (MES) versus non-MES (68). Moreover, NF1 loss that is frequently found in MES correlated with increased infiltration of macrophages/microglia. Venteicher et al. performed single-cell RNAseq experiments on GBMs with different IDH mutational status. In line with results from mouse models of GBM, it was reported that also in human GBM, the balance between microglia to macrophages shifts toward a higher representation of macrophage programs over microglia signatures (65). This effect was noted by downregulation of microglia core genes, such as CX3CR1, P2RY12, P2RY13, and SELPLG, concomitant with upregulation of macrophage-like signatures including increased expression of CD163, IFITM2, IFITM3, TAGLN2, F13A1, and TGFB1. Microglia and macrophages displayed signatures that reflected an inflammatory program consisting of cytokines (IL1, IL8, TNF), chemokines (CCL3, CCL4), NfkB-related genes as well as immediate early genes. In line with previous reports, expression analysis suggests that the GBM environment alters expression profiles of macrophages, thus reducing their transcriptional difference from microglia. Interestingly, the authors also identified a range of factors that correlated with increased macrophage infiltration. Of those 24 identified genes, three were components of the complement system (C1A, C1S, C4A) (65). Similarly, Darmanis et al. correlated gene expression within in the immune cell cluster (containing >95% macrophages/microglia and approximately 4.5% dendritic cells) with macrophage and microglia core genes to classify the cells into macrophages and microglia (67). Consistent with data obtained in GBM mouse models, it was shown that cells with macrophage-like signatures were found rather within tumor lesions, while microglia were localized at the tumor edge (67, 111). Interestingly, as previously proposed by Bowman et al., analysis of gene signatures revealed that more pro-inflammatory markers (e.g., CCL2, CCL4, TNF, IL6R, and IL1A/B) were expressed in the tumor periphery, whereas anti-inflammatory marker (e.g., IL1RN and TGFBI) were enriched in the tumor core (67, 112).

Taken together, while controversy remains on the extent of peripheral recruitment to GBM at different stages of tumor progression, there is accumulating evidence that the ontological

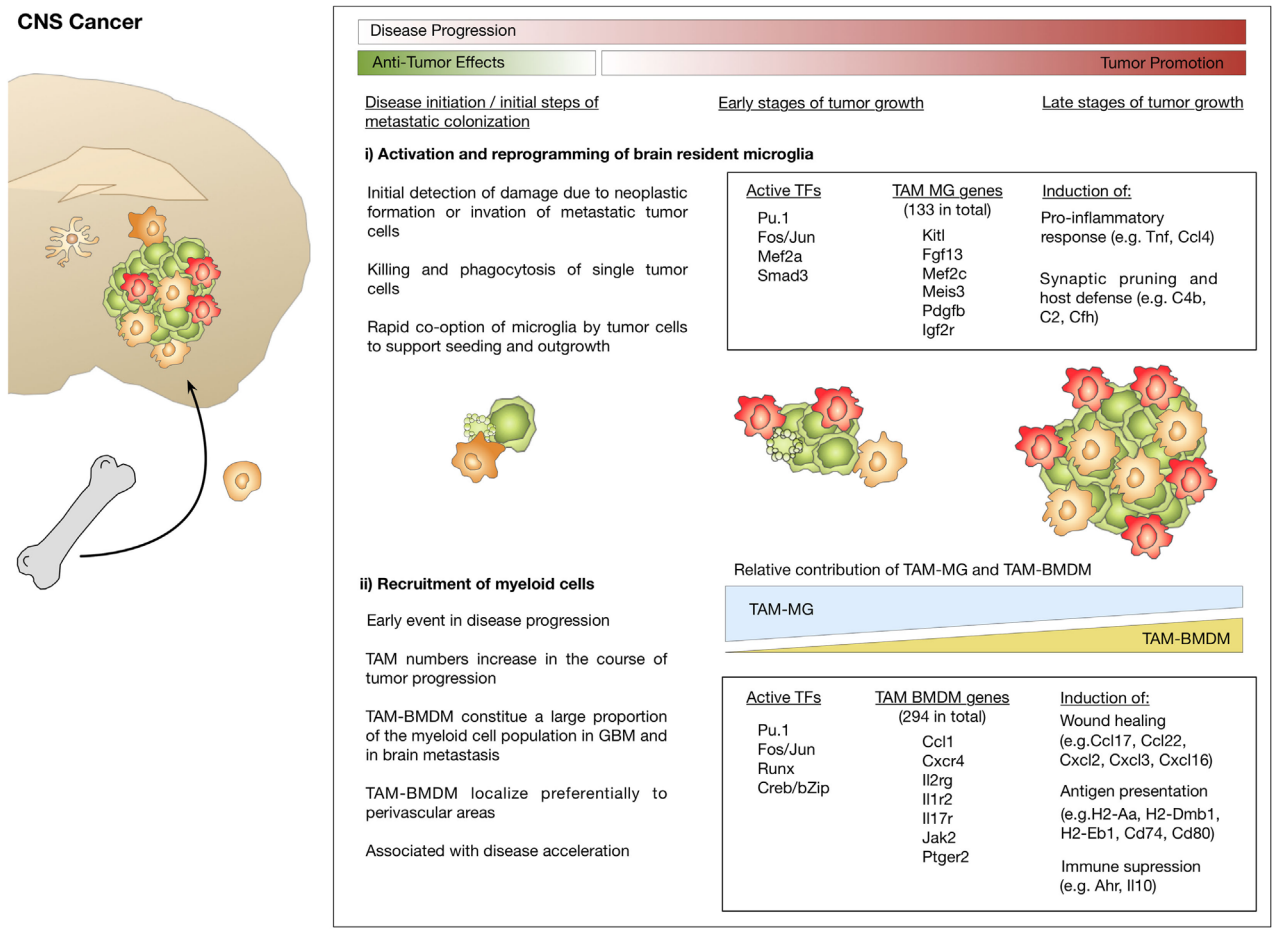

FIGURE 4 | Microglia and macrophage activation states and effector functions at different stages of disease progression in brain cancer. (i) Initial stages during neoplastic transformation in primary brain cancers or tumor cell seeding in brain metastasis are detected by microglia. As part of their role in host defense, microglia induce apoptosis in cancer cells. Tumor cells that escape microglia-mediated killing rapidly co-opt them and exploit their function to foster tumor growth. (ii) Brain tumor formation is associated with pronounced recruitment of macrophages from the periphery that starts at early stages and leads to disease acceleration. Transcriptomic analysis identified gene signatures that define tumor-associated microglia (TAM-MG) and macrophages (TAM-BMDM). The respective signatures indicate that TAM-MG rather induce pro-inflammatory responses and exert host defense functions while TAM-BMDMs were associated with wound healing, antigen presentation, and immune suppression.

origin of myeloid cells in brain cancers impacts the nature of genetic programs that are induced. Tumor-specific education of myeloid cells is expected to determine their effector functions during disease progression.

Lessons Learnt From Neurodegenerative Disorders and Brain Cancers

A series of recent studies utilized in depth transcriptomic analysis to molecularly define myeloid subpopulations at different stages of disease progression. However, a systematic analysis with the aim to interrelate the findings from different datasets has been missing. To close this gap of knowledge, Friedman et al. performed a comprehensive meta-analysis of purified mouse CNS myeloid cell profiles from different conditions across multiple studies including ischemic, infectious, inflammatory, neoplastic, demyelinating, and neurodegenerative conditions (123). Importantly, it was noted that in all comparisons microglia/brain-associated myeloid cell enriched genes that distinguish them from myeloid cells/macrophages from the periphery were downregulated. It appears that in response to any perturbation, including normal aging, the genes that separate microglia from other macrophages and are, thus, likely involved in microglia function, show reduced expression. Friedman et al. identified several modules that are shared across pathological conditions, including the neurodegeneration-associated module with high similarities to DAM (92, 123). In addition to the DAM-like population, a unique microglia subset that expressed an interferon-related gene module was identified. This module showed increased representation with progressive β-amyloid pathology. This is in line with the distinct clusters observed in the CKp25 model that showed enrichment of interferon-responding genes and likely reflect subpopulations depending on exposure to type 1 or type 2 interferons (93, 123). Moreover, the authors compared data sets from mouse models with human samples to evaluate the translational capacity of the

experimental data. Compared to mouse models, a greater range of cell-type variability was observed in the comparison of healthy and diseased human tissues, indicating that CNS inflammation in human disease is more pronounced than in commonly used mouse models (123).

Taken together, data obtained from studies on neurodegenerative disorders and brain cancers suggest that there is considerable overlap in certain tissue-specific gene signatures that are induced within the brain environment in response to disturbance of tissue homeostasis. This is apparent in the phenomenon that gene signatures in microglia and macrophages become more similar once both cell types are present in the brain (123). Moreover, a number of genes or pathways were found to be similarly dysregulated in neurodegenerative disorders and brain cancers and there is evidence of dichotomous roles of myeloid subpopulations/activation states under multiple pathological conditions. At early stages, myeloid cells are considered as protective cells that preserve tissue integrity by scavenging debris or eliminating intruders as part of their host defense mechanism. Advanced disease stages are rather associated with detrimental effects that cause tissue damage, neuronal loss and promote tumor growth (**Figures 3** and **4**) (99, 124). Neoplastic formation leads to a more rapid switch from beneficial to damaging effects and the extent of recruitment from the periphery appears to be more pronounced in brain cancers compared to neurodegeneration. It is increasingly recognized that tumor cells rapidly co-opt stromal cells and functionally reprogram their environment to generate a cancer permissive niche to foster tumor growth (124, 125). In this process, tumor cells are known to exploit housekeeping functions of stromal cells, such as host defense or wound healing mechanisms for their own benefit (126, 127). By contrast, recruitment of peripheral myeloid cells in neurodegenerative diseases such as AD appears to occur at later stages of disease progression and to a lesser extent. Protective programs of microglia have been shown to be preserved for an extended period during disease progression and to contribute in limiting disease propagation. However, at late disease stages, microglia function is no longer sufficient to prevent detrimental pathological events (99). Moreover, there is increasing evidence that dysregulation of certain pathways, e.g., the Trem2–Apoe pathway, represent a switch from protective to damaging effects (96).

As discussed in more detail in the next paragraph, detailed mechanistic insight is critical to develop therapeutic strategies that are targeted against aberrant functions of individual subpopulations at defined stages of disease progression as otherwise physiologically essential functions or protective programs might be blocked which consequently results in minimal therapeutic efficacy and adverse effects.

PERSPECTIVES FOR THERAPIES TARGETING MICROGLIA/MACROPHAGES IN NEURODEGENERATIVE DISEASE AND BRAIN CANCER

Macrophages/microglia-targeted therapies are emerging in the field of neurodegenerative disorders and cancer (128, 129). The rationale for environment-targeted therapies is based on high abundance of stromal cells in different pathologies as well as their critical impact on disease outcome. Moreover, it was reasoned that the risk for acquired resistance is lower in genetically stable non-malignant cells, compared to genetically instable cancer cells. Recent analysis of the phenotypic and functional heterogeneity of macrophage/microglia subpopulations in neurodegeneration and cancer clearly indicate that macrophage/microglia-targeted therapies have to be based on their disease-associated specificities to achieve high therapeutic efficacy without inducing adverse effects. A variety of macrophage/microglia-targeted strategies have been tested in pre-clinical settings using genetic and pharmacological approaches. Several inhibitors have already entered phase 2 clinical trials (130). Most of those strategies aim at either blocking the recruitment of macrophages or depleting them (131). Given the importance of CSF1R downstream signaling for the differentiation and survival of macrophages and microglia, many studies tested the efficacy of blocking the ligands (CSF1 and IL34) or the receptor. However, most of these studies reported no or low efficacy when used as monotherapy, while combination of CSF1R inhibition with standard of care (e.g., chemotherapy or irradiation) led to synergistic anti-tumor effects in, e.g., glioma and breast cancer (132, 133). By contrast, Pyonteck et al. demonstrated that monotherapy with the CSF1R inhibitor BLZ945 resulted in improved survival and tumor regression in a model for proneural GBM. CSF1R inhibition in this model did not result in depletion of TAMs. Instead, TAMs showed reduced expression of several M2-like markers. The authors concluded that CSF1R-induced depolarization of TAMs might be more efficient than depletion of TAMs (101, 131). The same group recently demonstrated that long-term CSF1R inhibitor treatment led to acquired resistance driven by a compensatory IGF1–IGF1R signaling loop between macrophages and tumor cells, resulting in enhanced glioma cell survival and invasion (134). CSF1R inhibitors are currently in clinical trials to test their efficacy in GBM patients. The clinical trial using the CSF1R inhibitor PLX3397 in recurrent GBM (NCT01349036) was recently completed. PLX3397 was well tolerated but showed no efficacy in the recruited patient cohort (130). Additional studies that test CSF1R inhibitors in combination with standard of care or immune therapy are currently ongoing [e.g., BLZ945 with PRD001 anti-programed cell death-1 (PD1) in solid tumors including recurrent GBM (NCT02829723) and PLX3397 with temozolomide and radiotherapy in newly diagnosed GBM (NCT01790503)].

Colony stimulating factor 1 receptor inhibition was also tested in models for neurodegenerative disease to limit damaging neuroinflammation at disease end stage. Elmore et al. depleted virtually the entire microglia pool using the CSF1R/c-KIT inhibitor PLX3397 with no impairment of behavior and cognition. After withdrawal of the inhibitor, microglia rapidly repopulated the brain, returning to normal numbers within two weeks. Replenishment of microglia after CSF1R inhibition occurred from nestin$^+$ progenitor cells that induced expression of microglia-associated genes such as Iba1, Cx3cr1, Tmem119, Siglech, Pu.1, and Trem2 (135). Repopulating microglia were shown to be functional and responsive to inflammatory challenge similar to resident microglia (136). Hence, CSF1R-mediated

microglia depletion might provide a powerful tool to resolve tissue destructive inflammation. Using the selective CSF1R inhibitor PLX5562, it was demonstrated that treatment with lower doses (leading to 30% depletion) strongly reduced microglia accumulation at amyloid plaques in the 3xTg-AD model. While plaque burden was not reduced, treatment led to improved cognition (137). Interestingly, CSF1 signaling can be regulated by Trem2, which suggests that effects of Trem2 on microglia could in part be mediated by the CSF1 signaling cascade (82). Likewise, Trem2 deficiency resulted in reduced accumulation of microglia around amyloid plaques. Krasemann et al. demonstrated that the Trem2–Apoe pathway induces a switch from a homeostatic to neurodegenerative phenotype in microglia. It was, therefore, proposed that modulation of the neurodegenerative phenotype through targeting of the Trem2–Apoe pathway might allow restoring homeostatic microglia and treat neurodegenerative disorders (96).

The recent advances in our understanding of niche-, stage-, and activation state-dependent roles of microglia and macrophages in different brain malignancies certainly open new opportunities for therapeutic intervention. Stage- or population-specific gene signature might also serve as new prognostic biomarkers to identify high-risk patients based on inflammatory fingerprints. Thorough functional validation of candidate genes that are associated with dysregulated microglia/macrophage function is needed to identify druggable targets for therapies aiming at reverting disease-promoting into protective effects or to maintain beneficial house-keeping functions as proposed for the Trem2–Apoe pathway.

CONCLUDING REMARKS

The history of microglia in brain malignancies started almost a century ago with their initial description by Pio del Rio-Hortega. Their ontological origin and biological function in health and disease has been controversially discussed ever since. While in-depth analysis down to the single-cell level provided critical insight into the heterogeneity of microglia and their blood-borne counterparts, we are just at the beginning to understand how different subpopulations or activation states regulate CNS homeostasis at steady state and how aberrant functions affect disease progression. Further investigation of the mechanisms that drive microglia and macrophage dysregulation will hopefully provide scientific rationale for the development of novel targeted therapies that provide better treatment options for patients to improve prognosis and quality of life.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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In Search for Reliable Markers of Glioma-Induced Polarization of Microglia

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Immune cells accumulating in the microenvironment of malignant tumors are tumor educated and contribute to its growth, progression, and evasion of antitumor immune responses. Glioblastoma (GBM), the common and most malignant primary brain tumor in adults, shows considerable accumulation of resident microglia and peripheral macrophages, and their polarization into tumor-supporting cells. There are controversies regarding a functional phenotype of glioma-associated microglia/macrophages (GAMs) due to a lack of consistent markers. Previous categorization of GAM polarization toward the M2 phenotype has been found inaccurate because of oversimplification of highly complex and heterogeneous responses. In this study, we characterized functional responses and gene expression in mouse and human microglial cultures exposed to fresh conditioned media [glioma-conditioned medium (GCM)] from human U87 and LN18 glioma cells. Functional analyses revealed mutual communication reflected by strong stimulation of glioma invasion by microglial cells and increased microglial phagocytosis after GCM treatment. To define transcriptomic markers of GCM-activated microglia, we performed selected and global gene expression analyses of stimulated microglial cells. We found activated pathways associated with immune evasion and TGF signaling. We performed computational comparison of the expression patterns of GAMs from human GBMs and rodent experimental gliomas to select genes consistently changed in different datasets. The analyses of marker genes in GAMs from different experimental models and clinical samples revealed only a small set of common genes, which reflects variegated responses in clinical and experimental settings. *Tgm2* and *Gpnmb* were the only two genes common in the analyzed data sets. We discuss potential sources of the observed differences and stress a great need for definitive elucidation of a functional state of GAMs.

Keywords: microglia, glioma, functional phenotype, transcriptomics, glioma-associated microglia/macrophages

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INTRODUCTION

Tumor microenvironment consists of various non-neoplastic cells that play an important role in tumor growth, progression, and immune response evasion. In glioblastoma (GBM), one of the most aggressive and prevalent primary brain malignancy in adults, tumor-infiltrating microglia and peripheral macrophages are major immune cell population within the tumor. Glioma-associated microglia/macrophages (GAMs) account for up to 30% of tumor mass in human GBMs (1–3) and in experimental glioma models (4–7). Glioma cells secrete various cytokines and chemokines acting as chemoattractants and polarizing factors on the resident microglia [for reviews, see Ref. (8, 9)].

Their role in glioma progression and immunosuppression has been shown in different experimental glioma models (10, 11). Pharmacological or genetic ablation of GAMs reduced tumor growth in mice experimental models of glioma (10, 12, 13). While controversy remains on the magnitude of peripheral macrophage recruitment to gliomas at different steps of tumor progression, there is growing evidence that tumor-specific education of GAMs is expected to determine their effector functions during tumor progression.

In tumor microenvironment, infiltrating macrophages adopt different activation states between antitumor M1 and protumor M2 phenotypes, and these functional phenotypes are defined by differential expression of surface markers, secreted cytokines, and roles in immunoregulation (14). M2 macrophages express frequently genes involved in tissue repair and resolution and have immunosuppressive and immunoregulatory properties. Clinical and mouse model data correlate the accumulation of macrophages with protumor activities. To define a functional phenotype of GAMs *in vivo*, these cells (usually defined as $CD11b^+$ cells) have been isolated using immunomagnetic beads or immunosorting and subjected to global gene expression profiling (2, 3, 5, 6). Most of those studies used microarray gene expression profiling, whereas RNA-seq was used to profile $CD11b^+$ in human samples. The resulting definitions of transcriptomic-based functional phenotypes of GAMs from human and experimental rodent gliomas are conflicting and indicate either a mixture of M1/M2 phenotypes (3, 5), the M0 phenotype (2), or the M2 phenotype (6). Categorization of GAM polarization using M1/M2 markers reported for peripheral macrophages (14) has been found inaccurate to describe discrete states due to oversimplification of highly complex and heterogeneous responses. There is no consensus and reliable gene expression-based markers.

We have previously characterized rat primary microglia cultures responses to rat C6 glioma-conditioned medium (GCM) and found that factors secreted by glioma cells induce the proinvasive, immunosuppressive polarization of microglia (15, 16). Selected genes were upregulated in cultured microglia, and their expression was also increased in GAMs isolated as $CD11b^+$ from C6 gliomas in rats (6). While animal models are a better tool to recreate complex cell–cell interactions between a tumor and its microenvironment, mechanistic analyses of events underlying activation are feasible in cell co-cultures. In this study, we characterized interactions between human glioma and microglial cells and defined gene expression profiles in search for markers of microglia polarization. In addition, we re-evaluated published datasets consisting of differentially expressed genes in GAMs from rat and mouse gliomas, and human GBM samples to identify overlapping or common genes. As a little commonality has been found, we discuss potential sources of variability and prospects of finding reliable markers.

MATERIALS AND METHODS

Microglial Cultures

Primary microglial cultures were prepared from cerebral cortices of P0–P2 old C57BL/6J mice. Following stripping off the meninges and enzymatic brain dissociation the cells were collected and seeded onto the culture flasks. After 48 h, cell cultures were washed three times with phosphate-buffered saline (PBS) to remove debris. Primary cultures were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, MD, USA). Following establishment of glial culture monolayer, microglial cells were isolated by gentle shaking for 1 h at 100 RPM at 37°C. Microglial cells were then collected by centrifugation, counted, checked for viability, and seeded at density 60×10^4 onto 60-mm plastic dishes (for non-adherent cultures). Microglial cultures were used for experiments 48 h after seeding.

Human immortalized SV40 microglia (Applied Biological Materials Inc.) were cultured in Prigrow III Medium (Applied Biological Materials Inc.) supplemented with 10% fetal bovine serum (Gibco, MD, USA) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) in a humidified atmosphere of CO_2/air at 37°C. Mouse microglial BV2 cells were cultured in DMEM with GlutaMAX™ (DMEM GlutaMAX™) supplemented with 2% fetal bovine serum (Gibco, MD, USA) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37°C (Heraeus, Hanau, Germany).

Glioma Cell Cultures

Human GBM cell lines LN18, U87-MG (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, MD, USA) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37°C (Heraeus, Hanau, Germany).

Patient-derived glioma cell cultures WG4 (GBM WHO grade IV) and IPIN20160420 (GBM WHO grade IV) were developed as previously described (17). Cells were cultured in DMEM/Nutrient Mixture F-12, GlutaMAX™ medium (DMEM/F-12, GlutaMAX™) supplemented with 10% fetal bovine serum (FBS, Gibco, MD, USA) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37°C (Heraeus, Hanau, Germany).

GCM and Microglia Treatment

Human glioma cells were seeded onto 60-mm dish at density 75×10^4 in DMEM or DMEM/F12, GlutaMAX™ supplemented with 10% fetal calf serum, and 100 U/mL penicillin and 100 µg/mL streptomycin. Following 24 h, the medium was changed to a microglia culture medium (DMEM or Prigrow III Medium 10% FBS with antibiotics), and glioma cells were cultured for another 24 h. Fresh GCM was collected and used to treat microglial cells. Cells exposed to GCM were collected after 3 and 6 h.

Functional Tests on Microglia

Morphological changes were assessed in microglial cultures using a co-culture system. Microglial cells were seeded onto round glass cover slips at 8×10^4/well in a 24-well plate and incubated for 48 h. Glioma cells were seeded into the Falcon cell culture inserts with 0.4-µm pores (Falcon #353095) at 6×10^4/insert. After 24 h, the inserts were transferred into the plate with microglial cells and co-cultured for 48 h. Microglial cells were fixed with 2% paraformaldehyde in PBS, permeabilized with 0.1% TRITON X-100,

and stained for 30 min at room temperature using Rhodamine–Phalloidin (1:1,000 in PBS). Cells were co-stained with DAPI (4′,6-diamidino-2-phenylindole; 0.001 mg/mL, Sigma) to visualize nuclei. Pictures were acquired using fluorescence microscope (Leica DM4000B, 40× objective). Images were analyzed using ImageJ software, and a ratio of Phalloidin stained area versus DAPI was used for a statistical analysis of cell size.

Phagocytic activity of microglia was assessed using flow cytometry. Microglial cells seeded onto 60-mm dishes and following 48 h, cells were treated for 24 h with fresh human GCM or LPS 100 ng/mL. Fluorescent latex beads were added (50 beads/cell; Sigma L3030) 3 h before read out. Cells were gently scrapped in cold PBS, washed twice and fluorescence was determined by FACSCalibur Flow Cytometer (BD Biosciences). In one run, 10,000 events were collected per sample and a mean fluorescence intensity (MFI) was calculated.

Gene Expression Analysis

RNA was isolated using RNeasy Mini Kit (QIAGEN, USA), followed by quality and quantity assessment using NanoDrop 2000 Spectrophotometer (Thermo Fisher, USA), and samples were stored at −80°C. RT-PCR was performed using SuperScript III Reverse Transcriptase (Invitrogen, USA) on 500 ng of total RNA and stored at −20°C. Gene expression profiling was performed by quantitative real-time PCR using 10 ng of cDNA in duplicates, using FAST SYBR Green PCR MasterMix (Life Technologies, USA) in 10 μL reaction with a listed set of primers (Table S1 in Supplementary Material). Amplified product was normalized to the endogenous expression of *18S*, and represented as delta Ct values.

Gene expression profiling of human SV40 microglia stimulated with GCM from human glioma LN18 and U87-MG cells was performed with GeneQuery™ Human Microglial Sensome qPCR Array (# GQH-MGS, ScienCell). GeneQuery™ qPCR array is a 96-well plate pre-made format; it contains in each well one validated primer set that recognizes and efficiently amplifies a specific target gene's cDNA. The primer set recognizes all known transcript variants of the target gene and amplifies only one gene. The array contains eight control genes. The annealing temperature in qPCR analysis was 65°C (with 2 mM Mg^{2+} and no DMSO). Gene expression profiling was performed by qPCR using 10 ng of cDNA from assorted samples from three duplicates for each group (untreated, GCM U87-MG, GCM LN18). The expression of selected genes were tested by qPCR with commercial primers for the following genes *P2RY12, SIGLEC1, SELPLG, IFITM5, PTAFR, TNFRSF1B*, and *GPR84*, purchased from ScienCell Research Laboratories (sequences undisclosed). Each primer set provided lyophilized and ready to use.

Global gene expression analysis using Affymetrix MG-430 PM Strip system (Affymetrix, USA) using GCM treated primary microglia for 6 h ($n = 3$). Samples were run on Bioanalyzer RNA 6000 Pico kit (Agilent, USA) to assess RNA integrity and concentration. The protocol was followed according to the manufacture's manual using 100 ng of total RNA and samples with RIN > 9.5. Hybridization was performed at 45°C for 16 h and fluidics, and imaging was done as described in the GeneAtlas System User's Guide (P/N 08-0246). All samples were run at the same time. Data were analyzed using R Studio software and Bioconductor package, and data were deposited into GEO Repository (GSE113370).

Invasion Assay

Microglial cells were plated onto 24-well plates at the density of 4×10^4. After 24 h, the invasion assay was performed using tissue culture inserts (6.5 mm Transwell® with 8.0 μm Pore Polycarbonate Membrane Insert, Corning, NY, USA) coated with the Growth Factor Reduced Matrigel™ Matrix (BD Biosciences, San Diego. CA, USA). 50 μL of the Matrigel™ Matrix (1 mg/mL) diluted in fresh DMEM medium was dried under sterile conditions (37°C) for 5–6 h. The medium was replaced to fresh one 1 h before seeding the glioma cells into inserts (to a medium for microglial cultures). The LN18, U87-MG, WG4, and IPIN20160420 cells were seeded at a density of 2×10^4/insert on matrigel-covered membrane in serum-reduced medium (2% FBS). The cultures were placed in a 37°C incubator with humidified air containing 5% CO_2. After 18 h, cells were fixed in ice-cold methanol and cell nuclei stained with DAPI (0.001 mg/mL, Sigma). The membranes from Transwell® inserts were cut out, and images were acquired using fluorescence microscope (Leica DM4000B, 10× objective) of the five independent fields (bottom, top, left, right side, and a center). Numbers of migrating cell nuclei were counted using ImageJ software. All experiments were performed at least three times, in duplicates.

Statistical Analysis

Each experiment was performed at least three times, on independent passages/cultures, at least in duplicates. Statistical analyses were performed using GraphPad Prism v6.01 (GraphPad Software, Inc., San Diego, CA, USA). The data were plotted as mean ± SD. Differences between the means of the treatments were evaluated using one-way analysis of variance (one-way ANOVA) followed by *post hoc* Dunnett's multiple comparison test or one-sided paired sample *t*-test (*p*-value) for two groups analysis. *p*-Values <0.05 were considered to be statistically significant and significance is marked as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Comparison of Different Gene Expression Data Sets

Mouse (E-MTAB-2660) and rat (E-MTAB-5050) microarray gene expression data were obtained from the Array Express database. Human RNA-seq expression profiles were obtained from GEO database (GSE80338). The raw microarray data were pre-processed using RMA as described (6). Annotation of probe sets was performed with information provided in the Ensembl database. For RNA-seq data, the already computed FPKM values were used. All data analyses were performed in the R statistical environment and relevant Bioconductor software.

RESULTS

Functional Analyses of Microglia Responses to Human Glioma Cells

Microglia can respond to various insults or environmental stimuli by changing its morphology, activating phagocytosis,

and modulating gene expression (16). Changes from thin/ramified to more amoeboid/round shapes are associated with functional activation *in vitro* and *in vivo* and are useful indicator of activated microglia. We used a co-culture system, in which cells are separated by inserts which allows only the exchange of soluble factors. Murine microglial cells were co-cultured with glioma cells to assess morphological changes induced by conditioned media from U87-MG and LN18 glioma cell cultures (**Figures 1A–D**). In the presence of glioma cells, we observed profound changes in morphology of microglia and transformation of bipolar cells to the amoeboid shape cells (**Figures 1B,C**). Insets show representative images of cells with changed morphology in higher magnification. We quantified percentages of amoeboid microglia under various conditions (**Figure 1D**). U87-MG glioma cells showed the stronger ability than LN18 to induce morphological changes of murine microglia that display more amoeboid/round shapes. Next, we employed flow cytometry to assess phagocytosis of fluorescent beads in primary microglial cultures upon the exposure to GCM from three glioma cultures or LPS, a potent immunomodulator. Phagocytosis was determined as mean fluorescence intensity (MFI) of cells. Graph shows FACS measurements from a representative experiment. There was a significant increase in phagocytosis following the treatment with GCM from primary GBM patient-derived cell cultures (IPIN20160420), and a consistent trend in the increase of phagocytosed beads in microglia treated with U87-MG GCM.

Interactions between microglia and human glioma cells were further investigated by a matrigel invasion assay in a co-culture system. We have previously demonstrated that the presence of rat microglia doubles glioma invasion (18, 19). First, we examined the ability of different microglial cells and other non-microglial cells to increase invasion of U87-MG glioma cells. As shown in the **Figure 1G**, all tested microglial cells: murine BV2 cells, primary murine microglial cultures, and human SV40 microglial cells significantly increased invasion of U87-MG glioma cells. Non-microglial cells, such as normal human astrocytes, human HEK-293 cells, and murine 3T3 fibroblasts, did not increase invasion of U87-MG glioma cells. As the primary microglial cultures produced most variable results, invasion assay experiments were performed on microglial BV2 cells. As the primary microglial cultures produced most variable results, invasion assay experiments were performed on microglial BV2 cells, and also for comparison on human SV40 cells (Figure S1 in Supplementary Material). We investigated the ability of BV2 microglial cells to support invasion of human U87-MG and LN18 glioma cells, as well as two patient-derived glioma cultures (IPIN20160420 and WG4; **Figure 1H**). Each glioma cell line had a specific invasion potential under basal conditions, so the increase of invasion in the presence of microglial cells was expressed as a fold change in relation to a cell line control performed for each treatment group. The results show a variable extent of dependence of glioma invasion on microglial cells. Three of four tested glioma cells showed increased in invasion in presence of murine microglial cells. The most prominent increase of invasion was observed in a case of U87-MG and WG4 cells, whereas IPIN20160420 showed moderate increase (**Figure 1H**). Altogether, our results show that both established and primary cultures of glioma interact in co-culture system, and U87-MG glioma cells exert strong and reproducible effect on murine microglia. Both murine and human microglial cells support invasion of U87 MG glioma cells, while astrocytes and other cells do not have such effect.

The Expression of Pre-Defined Marker Genes in Murine and Human Microglial Cells Treated With GCM From Human Glioma Cells

We have demonstrated the induction of certain genes in rat microglia cultures exposed for 6 h to GCM from C6 rat glioma cells (15, 16). Therefore, we sought to investigate whether those markers of microglia activation are modulated in murine microglia by GCM from human glioma cells. Primary murine microglia cultures were treated with fresh GCM from LN18 and U87-MG human glioma cells for 3 and 6 h. The expression of *Mmp14*, *cMyc*, *Arg1*, *Ifnb1*, *Cxcl14*, *Irf7*, and *Smad7* was assessed with qRT-PCR, and the results are plotted as delta Ct values relative to the endogenous *18S* expression (**Figure 2**). Increases in the *Mmp14* and *Arg1* expression were detected in microglia exposed to GCM from U87-MG cells for 3 h. Small increases of *Arg1*, *Cxcl14*, and *Smad7* mRNA levels at 6 h were observed; however, due to large variations between the biological repeats, these changes did not reach significance. None of the tested genes was significantly upregulated by GCM from LN18 glioma cells. The basal expression of selected genes varied between 3 and 6 h. The expression of endogenous *18S* was used as a reference for the amount of cDNA as its expression did not change following treatment.

We used GeneQuery™ Human Microglial Sensome qPCR Array examining 89 genes to perform gene expression profiling of human SV40 microglial cells polarized by GCM from human LN18 and U87-MG glioma cells. The results, presented as a heatmap, demonstrate moderate similarity in the profiles of upregulated genes in microglia stimulated with GCM U87-MG or LN18. The expression of genes strongly upregulated by either treatment is indicated by arrows and gene names (**Figure 3A**). The expression of selected, upregulated genes (incorporating *c-MYC, SMAD7,* and *MMP14*) was validated by qPCR in independent cultures of human SV40 microglial cells treated for 6 h with GCM and corroborated the qPCR Array results. A number of genes were significantly upregulated, and we observed the stronger effect after GCM LN18 treatment (**Figure 3B**).

Global Gene Expression Analysis in GCM-Treated Mice Microglia

As the preselected gene approach did not appear useful in identification of genes modulated during polarization of microglia by human glioma cells, to identify differentially expressed genes, we performed gene expression using Affymetrix arrays. Microarray analysis was performed on murine microglial cells exposed to fresh GCM from LN18 and U87-MG for 6 h. Volcano plots represent differentially expressed genes relative to untreated microglia (**Figure 4A**). Following the analysis, 36 unique genes were found

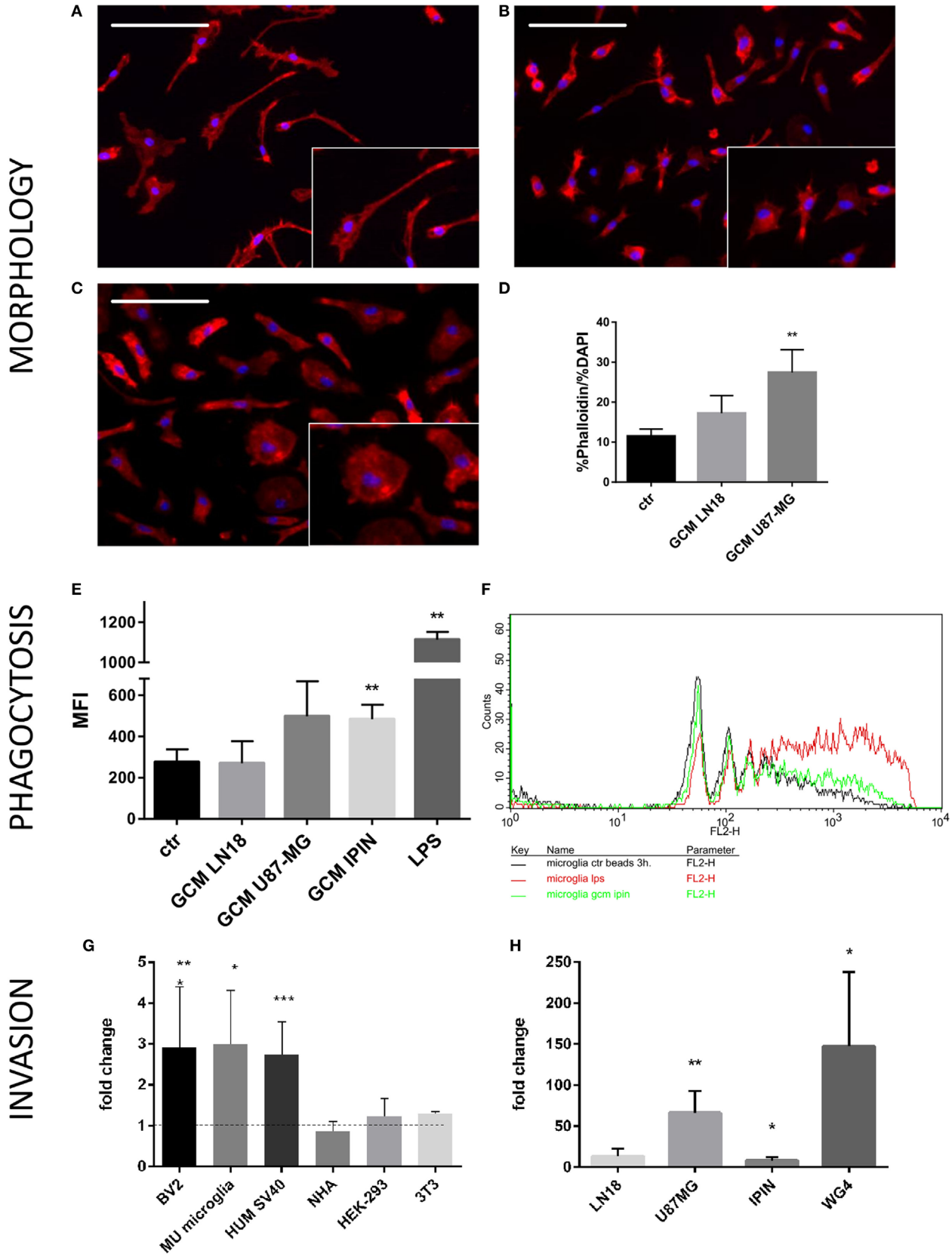


FIGURE 1 | Continued

FIGURE 1 | Functional analyses of glioma-induced polarization of murine microglia. Primary murine microglia cultures were co-cultured with human U87-MG or LN18 glioma cells. **(A–C)** Representative images show morphological changes induced in primary murine microglia cultures following co-culture with human U87-MG or LN18 glioma cells. Morphological alterations were visualized by F-actin staining; cell nuclei were co-stained with DAPI. Insets show in higher magnification numerous microglia with amoeboid shape in co-cultures with U87-MG cells. **(D)** Changes were quantified by ratio of percentage of Phalloidin staining to percentage of DAPI staining that is proportional to cell size. **(E,F)** Murine microglia were treated for 24 h with conditioned media [glioma-conditioned medium (GCM)] from human U87-MG, LN18, and IPIN glioma cells or LPS (100 ng/mL), incubated for 3 h with fluorescent beads and subsequently analyzed by flow cytometry. Phagocytosis of fluorescent beads in microglia is represented as mean fluorescence intensity (MFI); graph shows statistically significant groups **(F)** using one-way ANOVA with Dunnett's multiple comparisons test; $n \geq 3$. **(G)** Matrigel assay was performed to determine invasion of human U87-MG glioma cells in the presence of different microglial (BV2—immortalized murine microglia, Mu microglia—primary microglial cultures, SV40 immortalized human microglia) or non-microglial cells (NHA—normal human astrocytes, HEP293 cells, and NIH3T3 fibroblasts). Data are calculated as fold change in relation to basal invasion in the absence of microglia. Matrigel invasion data are calculated as means ± SD, $n \geq 3$ and were analyzed by one-sided paired sample *t*-test; $n \geq 3$. Differences at $p < 0.05$ were considered significant (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$). **(H)** Invasiveness of various human glioma cells (IPIN and WG4 are primary patient-derived glioma cultures) is increased to different extent in the presence of BV2 murine microglial cells.

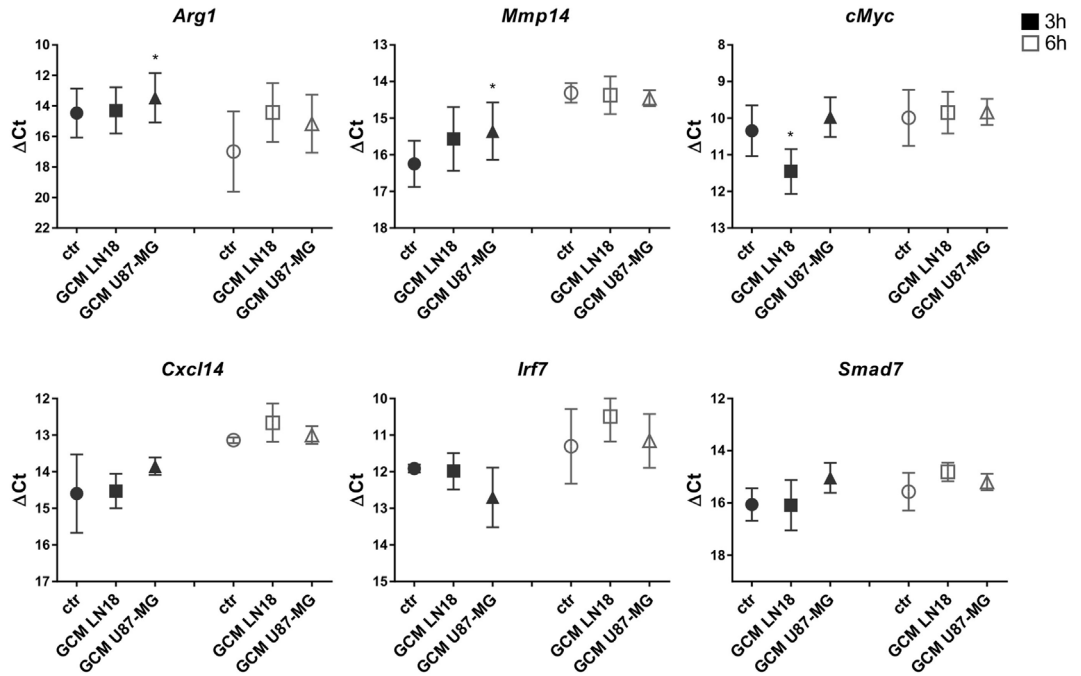


FIGURE 2 | The expression of selected genes in glioma-conditioned medium (GCM) stimulated primary murine microglia cultures. Gene expression was determined by qRT-PCR in microglial cultures left untreated (circles), treated with GCM from LN18 (squares), or U87-MG (triangles) for 3 h (black) and 6 h (white). Data are shown as delta Ct values relative to *18S* expression. Assessment of statistical significance was performed using one-way ANOVA test, followed by Dunnett's multiple comparison test. The results are calculated as means ± SD, $n \geq 3$. Differences at $p < 0.05$ were considered as significant (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$).

to be significantly differentially expressed, with 20 genes common in both groups. There were 15 uniquely expressed genes in GCM LN18 and only 1 in GCM U87-MG (*Acss1*) stimulated microglia. Overall, the expression of genes was similar in LN18 and U87 GCM-treated microglia (**Figure 4B**). We performed validation of selected genes by qPCR and found five of six upregulated by GCM from two cell lines (Figure S2 in Supplementary Material).

Genes upregulated in both groups are associated with immune response, chemotaxis, and signal transduction regulation as defined by Gene Ontology (GO) functions (**Figure 4C**). Interestingly, we found two upregulated genes that were related to transforming growth factor beta (TGFβ) signaling. Western blot analysis for a phosphorylated Smad2, an active component of TGFβ signaling pathway, was performed on total extracts from microglia polarized with GCM from glioma cells. We found the increased levels of active, phosphorylated Smad2 proteins accumulating at 6 h after GCM treatment (Figure S3 in Supplementary Material).

Tgm2 and *Gpnmb* Are Universal Markers of GAMs

Our results show surprisingly low commonality in transcriptomic responses in different models of glioma-microglia interactions. Therefore, we performed reassessment of publicly available datasets for genome-wide analysis of gene expression in GAMs isolated from mouse (5) and rat gliomas (6), and compared the results to human GAMs data sets (2, 3).

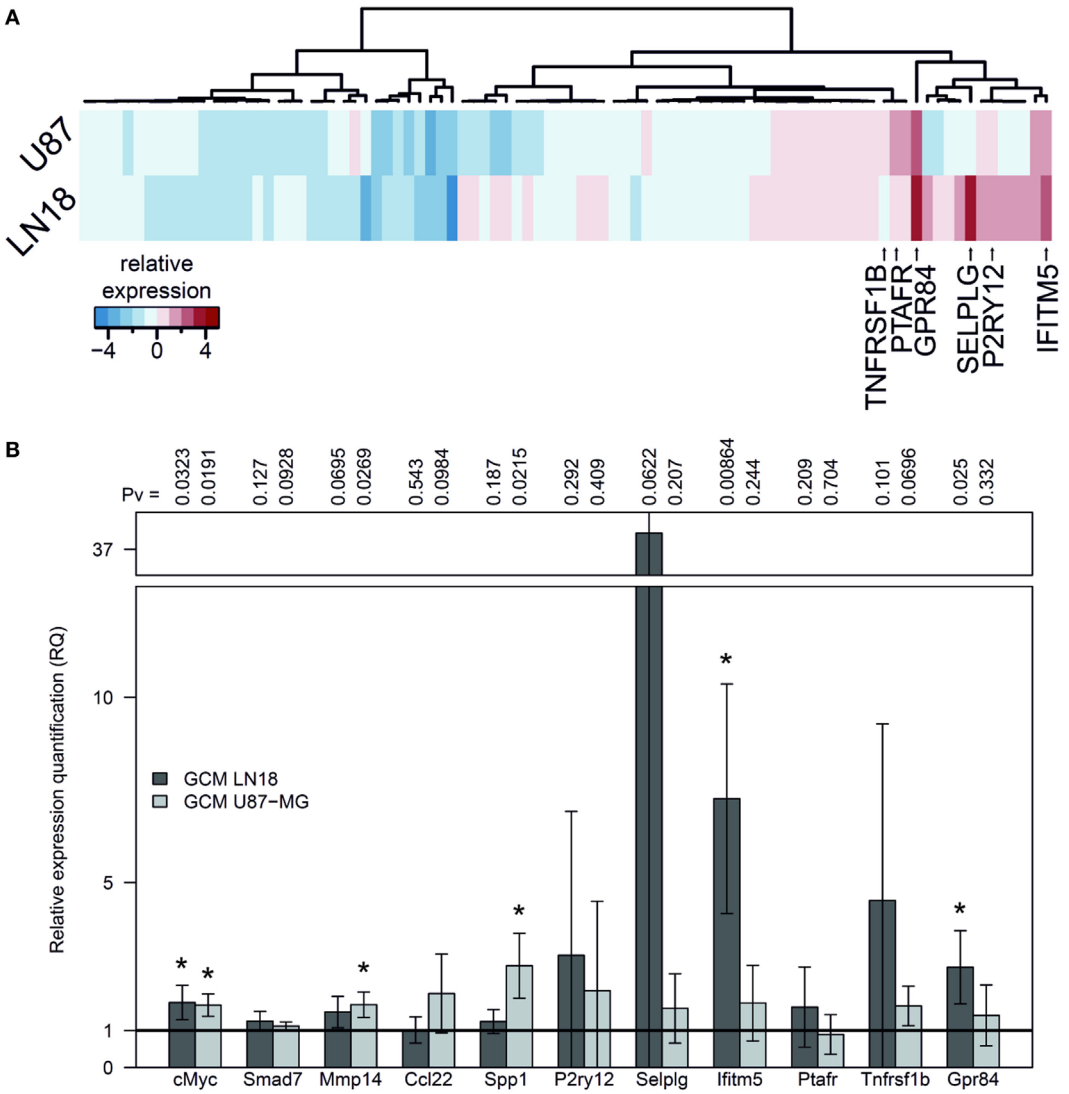
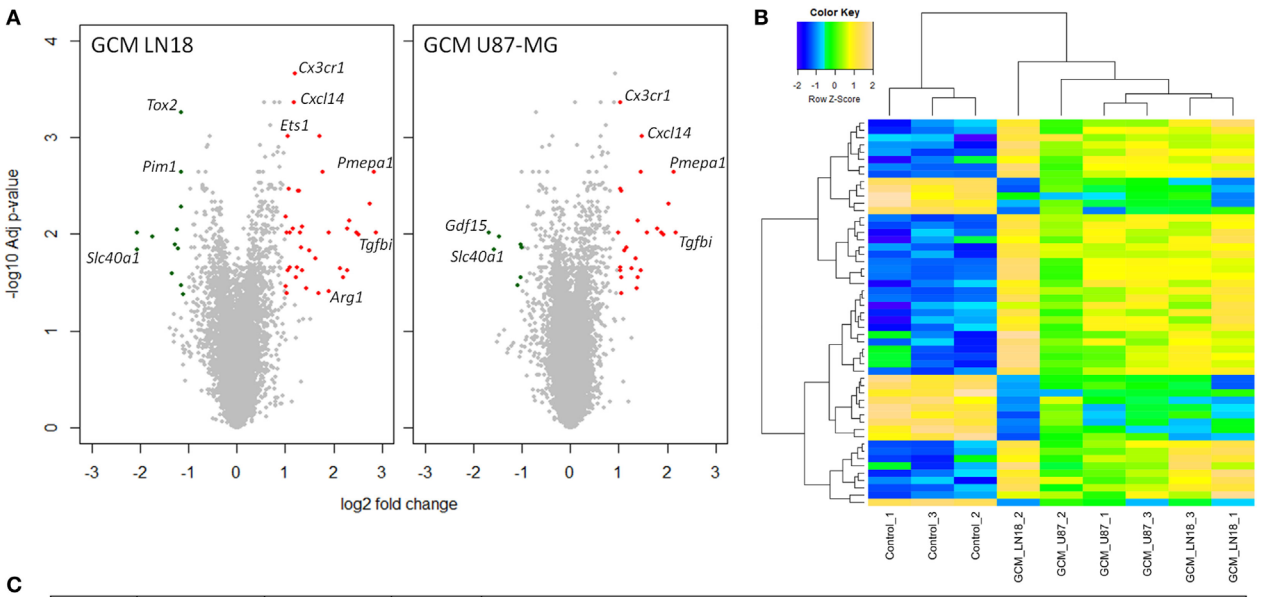


FIGURE 3 | Gene expression in human SV40 microglia polarized by factors secreted by human glioma cells. **(A)** Human Microglial Sensome Gene expression profiling in human SV40 microglia treated by glioma-conditioned medium (GCM) from human LN18 and U87-MG glioma cells was determined with GeneQuery™ Human Microglial Sensome qPCR Array 6 h after GCM addition. The analysis was performed on assorted samples from three replicates for each group (untreated, stimulated with GCM U87-MG or LN18). Heatmap was generated to represent differentially expressed genes and samples. **(B)** The expression of selected genes (*cMYC, SMAD7, MMP14, CCL22,* and *SPP1*) was validated by qPCR in independent cultures of human SV40 microglia treated with GCM from U87-MG or LN18 cells for 6 h. The results are shown as relative quantification (RQ) of GCM stimulated samples compared with untreated controls. Assessment of statistical significance was performed using one-sample *t*-test. The results are calculated as means ± SD, $n \geq 3$. Differences at $p < 0.05$ were considered as significant (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$).

Although we found hundreds of upregulated genes in either mouse or rat GAMs (**Figure 5**), the correlation analysis computed using expression changes of orthologs in GAMs versus control microglia did not point to any model which more closely mimics gene expression levels in human GAMs (Figure S4 in Supplementary Material). Nevertheless, we observed that expression changes are more prominent in a mouse model (**Figures 5A,B**). We identified 890 genes with higher expression in mouse GAMs, when compared with control microglia samples, and 621 of such genes in rat GAMs; 277 of the identified genes were common for both models (**Figure 5C**). GO analysis revealed that out four of five top enriched GO terms are common for mouse and rat models (**Figures 5D,E**). Interestingly, GO terms identified only for mouse model were connected mostly with cellular responses, while GO terms identified in a rat model were mainly connected with chromatin and cell cycle. In summary, these results show that despite some similarities, the mouse and rat models represent different pathways of GAM activation.

To determine whether any universal GAM marker might be identified, we set up a comparative analysis of four publically available data sets: (a) MACS sorted $CD14^+$ from human GBM



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Gene	GCM LN18 (FC)	GCM U87-MG (FC)	Adj p.val	GO biological function
Cxcl14	2.3	2.0	4.34E-04	immune response, cell chemotaxis
Olfml3	3.2	2.8	9.77E-04	multicellular organismal development
Pmepa1	7.0	4.3	2.28E-03	negative regulation of signal transduction, negative regulation of transforming growth factor beta receptor signaling pathway
Ctla2a/b	3.5	2.8	2.28E-03	regulation of regulatory T cell differentiation, negative regulation of inflammatory response
Lrrc16a	4.9	3.5	7.34E-03	positive regulation of cell migration, positive regulation of actin filament polymerization
Tgfbi	4.9	3.5	8.73E-03	cell adhesion, extracellular matrix organization, chondrocyte differentiation, angiogenesis
Ch25h	2.1	2.0	2.23E-02	lipid metabolic process, oxidation-reduction process
Tgm2	4.3	2.5	2.26E-02	positive regulation of I-κB kinase/NF-κB signaling , positive regulation of inflammatory response , apoptotic cell clearance
Gatm	2.5	2.0	2.38E-02	creatine biosynthetic process, tissue regeneration, response to oxidative stress

FIGURE 4 | Global gene analysis in primary microglia cultures stimulated with glioma-conditioned medium (GCM) from human glioma cells. Primary microglia cultures were treated for 6 h with GCM. Total RNAs were isolated and subjected to Affymetrix microarray analysis. Data were pre-processed as described in Section "Materials and Methods" and significantly regulated genes are depicted. **(A)** Volcano plots represent log2 fold change versus −log10 adjusted *p*-value in microglia treated with GCM from LN18 and U87-MG glioma cells; upregulated genes are marked in red; downregulated genes in green. **(B)** Heatmap was generated to show significantly differentially expressed genes and sample clustering. **(C)** Selected top significantly upregulated genes in microglia treated with GCM LN18 or U87-MG were listed in a table with fold change expression (FC), adjusted *p*-value, and associated Gene Ontology (GO) biological functions. Significance was assigned to adj *p*-val < 0.05.

versus postmortem tissue and low grade gliomas profiled with microarrays (2); (b) MACS sorted CD11b$^+$ from human GBM versus postmortem and epilepsy tissue analyzed by RNA-seq (3); (c) MACS sorted CD11b$^+$ from murine GL261 GBM profiled with microarrays (5); (d) FACS sorted CD11b$^+$/CD45^{low} from rat C6 gliomas profiled with microarrays (6). Those data were compared with the *in vitro* model presented here—primary murine microglia cultures stimulated with LN18 or U87-MG GCM and profiled with microarrays.

We have found that across all data sets, 129 genes are significantly upregulated (inclusion criteria $p < 0.05$, FC > 2) in at least 2 models, whereas only 4 genes were found to be upregulated in 3 models, and none appeared in 4 or more data sets (**Table 1**). Downregulated genes (inclusion criteria $p < 0.05$, FC < −2) showed nearly no overlap—only one gene was shared between two data sets. The lack of commonly downregulated genes could be caused by very low number of downregulated genes compared with upregulated across investigated data sets (27 down and 17 up in human CD14$^+$; 43 down and 292 up in human CD11b$^+$, 78 down and 539 up in murine CD11b$^+$; 36 down and 287 up in rat CD11b$^+$CD45^{low}). Therefore, in further analysis, we focused on the upregulated genes.

Interestingly, all of the four genes overexpressed in three data sets—*Tgm2, Gpnmb, Il2ra,* and *Ccr7* (**Table 1**), encode proteins that are expressed at the plasma membrane, which makes them good marker candidates. Transglutaminase 2 (Tgm2) was upregulated in human CD11b$^+$, murine CD11b$^+$, and murine microglia stimulated with LN18 GCM. Rat microglia (CD11b$^+$CD45^{low}) also expressed *Tgm2* at high level

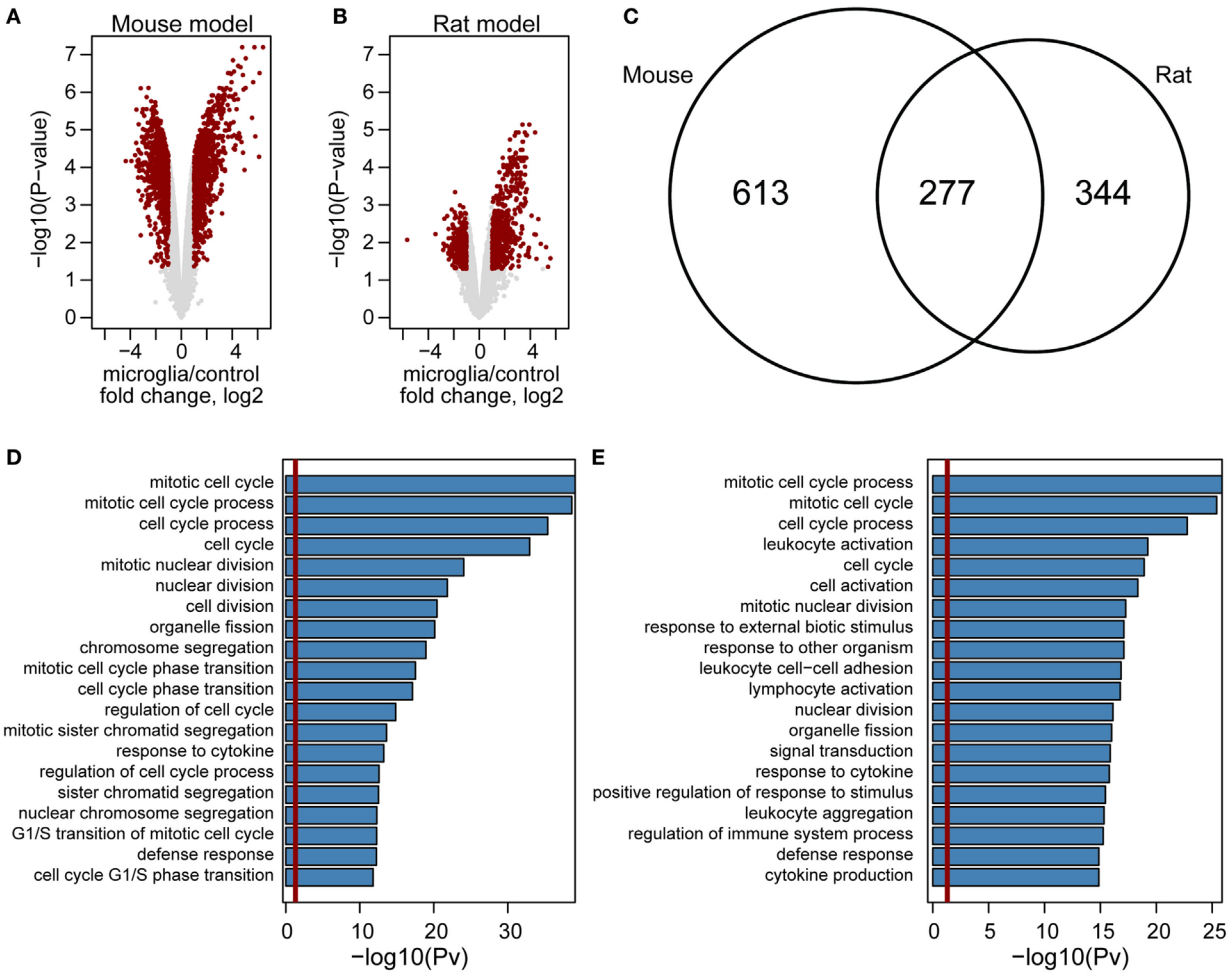

FIGURE 5 | Genome-wide comparison of gene expression in microglia in mouse and rat glioma models. Volcano plot of gene expression changes in glioma-associated microglia/macrophages from mouse **(A)** and rat **(B)** models. The data for all genes are plotted as log2 fold change between microglia and control samples versus the –log10 of the corresponding adjusted *p*-values. Genes selected as significantly different are highlighted as red dots. Venn diagram for overlap of genes upregulated in mouse (left) and rat (right) model **(C)**. Enrichment analysis of Gene Ontology terms within mouse **(D)** and rat **(E)** upregulated genes. Bar plots represent the statistical significance of the enrichment [–log10 (*p*-val)] **(D,E)**.

(FC = 8.12), although the upregulation was not significant ($p = 0.098$). Tgm2 is a cross-linking enzyme involved in matrix stabilization, which is necessary for effective phagocytosis of apoptotic cells by macrophages (20). The role of Tgm2 was also shown in tumorigenesis, as it is highly expressed by $CD44^{high}$ (stem cells marker) glioma tissue and Tgm2 inhibition lead to decrease of $CD44^{high}$ cells (21). In turn, upregulation of *Gpnmb* was found for all three investigated species (murine and human $CD11b^+$, and rat $CD11b^+CD45^{low}$). The importance of transmembrane glycoprotein NMB (Gpnmb) was already implied by Szulzewsky et al. (5), who showed its overexpression in mouse and human $CD11b^+$ GAMs, and demonstrated an association between increased *Gpnmb* expression and poor patient prognosis.

Interleukins and chemokines are cytokine families frequently overexpressed by GAMs in various glioma models. Nevertheless, we have found that only one member of each family—interleukin 2 receptor alpha (Il2ra) and C-C chemokine receptor type 7 (Ccr7) is overexpressed in three of five analyzed models. Importantly, in contrast to *Tgm2* and *Gpnmb,* overexpression of *Il2ra* and *Ccr7* was found only in human and murine GAMs and did not occur in either a rat experimental model or an *in vitro* model.

Enriched Functional Groups in GAMs

In addition to screening for a specific GAM marker, we performed a search for enriched GO biological processes. Enriched GO terms that contained genes represented in all data sets are presented in **Table 2** (for complete functional analysis results see Table S2 in Supplementary Material). Notably, out of 129 genes upregulated in at least 2 data sets, 68 genes were shared between human $CD11b^+$ and rat $CD11b^+CD45^{low}$ data sets, and only 27 and 22 genes between human $CD11b^+$ and murine $CD11b^+$, and rat $CD11b^+CD45^{low}$ and murine $CD11b^+$ data sets,

TABLE 1 | Top markers of glioma infiltrating microglia/macrophages.

	Human CD14$^+$		**Human CD11b$^+$**		**Murine CD11b$^+$**		**Rat CD11b$^+$CD45^{low}**		**Primary murine microglia stimulated with**			
									LN18 glioma-conditioned medium (GCM)		**U87 GCM**	
Gene ID	Gabrusiewicz et al. (2)		Szulzewsky et al. (3)		Szulzewsky et al. (5)		Gieryng et al. (6)		Walentynowicz et al.			
	FC	adj. *p*-value	FC	adj. *p*-value	FC	adj. *p*-value	FC	adj. *p*-value	FC	adj. *p*-value	FC	adj. *p*-value
Tgm2	–	–	**6.50**	4.60E−02	**6.94**	8.33E−04	**8.12**	9.80E−02*	**4.29**	2.60E−02	**2.46**	2.60E−02
Gpnmb	–	–	**8.71**	1.22E−06	**36.02**	2.61E−05	**11.54**	4.16E−02	–	–	**–**	–
Il2ra/rn		Upregulated	**4.44**	1.79E−07	**5.24**	2.77E−05						
Ccr7		Upregulated	**2.83**	3.05E−02	**3.75**	2.29E−03	–	–	–	–	–	–

Only genes that were upregulated in the minimum of three models were included.
**Raw data for Gabrusiewicz et al. (2) were not available, thus the gene expression is indicated based on a heatmap from Figure 6 (2).*

respectively. In addition, the most enriched GO term—mitotic cell cycle—contains mainly genes from the human CD11b$^+$ and rat CD11b$^+$CD45^{low} data sets.

This result is not biased by the size of data sets, since comparable numbers of genes were included for the analysis of human CD11b$^+$ and rat CD11b$^+$CD45^{low}, whereas the murine CD11b$^+$ data set was even bigger (**Table 2**). Therefore, we provide further evidence that the immune infiltrates of human GBMs share more similarities with rat C6 gliomas than with murine GL261 gliomas (6).

Gene Ontology term "Regulation of cell adhesion" consisted of genes which roles in the glioma progression were widely studied: *Il10, Mmp14,* and *Spp1*. Interestingly, upregulation of all of the three genes was found only for the murine CD11b$^+$ GAMs, *IL10* and *SPP1* were also found in CD14$^+$ human GAMs and *MMP14* in CD11b$^+$ human GAMs. In rat, CD11b$^+$CD45^{low} *Spp1* was also upregulated; however, the increase did not cross the significance threshold (logFC = 5.39, *p*-adj = 0.074), *Il10* and *Mmp14* were also represented in the rat data set, but no change was noted for those genes (Il10 logFC = 0.98, *p*-adj = 0.536; Mmp14 logFC = 1.27, *p*-adj = 0.03).

Other less known genes (*Lgals1/3, Ctla2a, Il2rn, Il1ra,* and *Prdm1*) play a role in T-cell activity and immune response regulation. Cytotoxic T-lymphocyte-associated protein 2 alpha (Ctl2a) is expressed by activated T-cells; however, its function has not been widely studied (22). Galectins are family of proteins regulating T-cell death, where Galectin 1 (Lgals1) is a strong inducer of T-cell apoptosis, and Galectin 3 (Lgals3) either blocks or induces T-cell death depending whether it occurs intra- or extracellularly (23). *Il1rn* is a gene encoding interleukin 1 receptor antagonist, which functions as an inhibitor of the inflammatory response mediators IL1α and IL1β, whereas *Il2ra* encodes an alpha chain of IL2 receptor and its homodimer form a low-affinity receptor. Positive regulatory domain I-binding factor 1 (Prdm1) in turn, is a transcription factor critical for production of IL-10 cytokine, which expression is restricted to antigen-exposed CD4$^+$ and CD8$^+$ T-cells (24, 25).

Six genes of chemokine family were found in "Cell chemotaxis" GO category; however, this group appeared to be relatively model specific. Human and murine GAMs (CD11b$^+$) shared upregulation of *Ccr7* and *Cxcl2,* murine and rat GAMs shared only *Cxcl10* overexpression. Chemokines unique for each model were human CD14b$^+$—*CCL13*; human CD11b$^+$—*CCL20*; murine CD11b$^+$—*Ccl8, Ccl11, Ccl17, Cxcl16, Cxcl4*; rat CD11b$^+$CD45^{low}—*Cxcl13*. Among genes of GO terms "Regulation of response to external stimulus" and "Blood vessel morphogenesis," we found *Ptgs2* encoding prostaglandin endoperoxide synthase 2 (COX-2), an enzyme producing inflammatory prostaglandins. *PTGS2* is overexpressed in many cancers and was shown to play role in enhancing tumor progression and angiogenesis [reviewed in Ref. (34)].

DISCUSSION

The concept of GAMs playing a key role in GBM pathobiology has been verified in recent studies that demonstrated reduction of glioma growth after microglia ablation or pharmacological inhibition (10, 12, 13). We could use this knowledge to steer GAMs from a tumor supportive and anti-inflammatory phenotype toward an antitumor phenotype. However, translation of such results to a clinic has been found difficult as clinical trials with molecules validated in murine models failed to be successful (26). We still need to separate roles of microglia and macrophages and identify signaling pathways underlying their polarization. Use of CD11bCD45 allows for separation of microglia and bone marrow-derived macrophages that infiltrate the tumor mass in rodent models but this cannot be achieved in human glioma samples, as CD11bCD45 discrimination is challenging in flow cytometry analysis.

We intended to understand mechanisms underlying polarization of microglia driven by factors secreted by human glioma cells in a controlled system. Murine microglia exposed to human glioma undergo morphological alterations, increased phagocytosis, and supported glioma invasion (**Figures 1A–H**). Several of those responses were mimicked by immortalized BV2 and human SV40 microglial cells. We found that microglia functional responses and gene expression profiles differ when exposed to different human glioma cells. Many previously described markers did not manifest in murine microglia polarized by human glioma cells. It is worth

TABLE 2 | Co-occurrence of genes from the same functional groups across all data sets.

Data set		Human CD14$^+$	Human CD11b$^+$	Murine CD11b$^+$	Rat CD11b$^+$CD45^{low}
Genes upregulated in minimum 2 sets/total number of upregulated genes in the set		11/17	96/292	60/539	88/287
Enriched Gene Ontology (GO) biological process	All	Gabrusiewicz et al. (2)	Szulzewsky et al. (3)	Szulzewsky et al. (5)	Gieryng et al. (6)
Mitotic cell cycle GO:0000278 adj. p-value 9.88×10^{-9}	*Bub1 Aurkb Bub1 Bub1b Ccnb1 Cdc45 Cdc6 Cdca8 Cdk1 Cdkn3 Cdt1 Cenpf Cep55 Chek1 Cit Clspn Dtl E2f7 E2f8 Espl1 Fanci Foxm1 Gpnmb Gpr132 Kif11 Kif18b Kif23 Kif4 Mad2l1 Mki67 Mybl2 Ncapg Ndc80 Nek2 Nuf2 Nusap1 Pim1 Rad51 Ska1 Ska3 Top2a Tpx2 Ube2c*		*Aurkb Bub1 Bub1b Ccnb1 Cdc45 Cdc6 Cdca8 Cdk1 Cdkn3 Cdt1 Cenpf Cep55 Chek1 Cit Clspn Dtl E2f7 E2f8 Espl1 Fanci Foxm1 Gpnmb Kif11 Kif18b Kif23 Mad2l1 Mki67 Mybl2 Ncapg Ndc80 Nek2 Nuf2 Nusap1 Rad51 Ska1 Ska3 Top2a Tpx2 Ube2c*	*Gpnmb Gpr132 Pim1*	*Aurkb Bub1 Bub1b Ccnb1 Cdc45 Cdc6 Cdca8 Cdk1 Cdkn3 Cdt1 Cenpf Cep55 Chek1 Cit Clspn Dtl E2f7 E2f8 Espl1 Fanci Foxm1 Gpnmb Gpr132 Kif11 Kif18b Kif23 Mad2l1 Mki67 Mybl2 Ncapg Ndc80 Nek2 Nuf2 Nusap1 Pim1 Rad51 Ska1 Ska3 Top2a Tpx2 Ube2c*
Regulation of cell adhesion GO:0030155 adj. p-value 2.25×10^{-2}	*Prdm1 Ccr7 Ctla2a Has2 Il10 Il1rn Il2ra Lgals1 Lgals3 Mmp14 Nrp1 Plau Ccl5 Spp1 Tgm2 Tnc Vcam1 Gpnmb*	*Ccl5 Ccr7 Il10 Il1rn Il2ra Spp1*	*Ccr7 Gpnmb Has2 Il2ra Lgals1 Lgals3 Mmp14 Nrp1 Plau Tgm2 Tnc*	*Ccl5 Ccr7 Ctla2a Gpnmb Has2 Il10 Il1rn Il2ra Lgals1 Mmp14 Prdm1 Spp1 Tgm2 Tnc Vcam1*	*Gpnmb Lgals3 Nrp1 Plau Prdm1 Vcam1*
Cell chemotaxis GO:0060326 adj. p-value 2.50×10^{-2}	*Ccl5 Ccl18 Ccr7 Cxcl10 Cxcl2 Cxcl9 Jaml Lgals3 Nrp1 Pde4b Spp1 Vcam1*	*Ccl5 Ccl18 Ccr7 Cxcl9 Spp1*	*Ccl18 Ccr7 Cxcl2 Lgals3 Nrp1*	*Ccl5 Ccr7 Cxcl10 Cxcl2 Cxcl9 Pde4b Spp1 Vcam1*	*Cxcl10 Lgals3 Nrp1 Pde4b Vcam1*
Regulation of response to external stimulus GO:0032101 adj. p-value 2.63×10^{-2}	*Acp5 Aoah Ccl5 Ccr7 Cd109 Ctla2a Cxcl10 Cxcl2 Cxcl9 Gbp4 Il10 Il2ra Nrp1 Plau Prdm1 Ptgs2 Tgm2 Usp18*	*Ccl5 Ccr7 Cxcl9 Il10 Il2ra Ptgs2*	*Acp5 Ccr7 Cd109 Cxcl2 Il2ra Nrp1 Plau Tgm2*	*Acp5 Ccl5 Ccr7 Cd109 Ctla2a Cxcl10 Cxcl2 Cxcl9 Gbp4 Il10 Il2ra Prdm1 Ptgs2 Tgm2 Usp18*	*Aoah Cxcl10 Gbp4 Nrp1 Plau Prdm1 Usp18*
Blood vessel morphogenesis GO:0048514 adj. p-value 5.44×10^{-2}	*Ahr Ccl5 Clic4 Cxcl10 E2f7 E2f8 Foxm1 Has2 Hif1a Lgals3 Nrp1 Plau Prdm1 Prrx1 Ptgs*	*Ccl5 Hif1a Ptgs2*	*Ahr E2f7 E2f8 Foxm1 Has2 Lgals3 Nrp1 Plau Prrx1*	*Ahr Ccl5 Clic4 Cxcl10 Has2 Hif1a Prdm1 Prrx1 Ptgs2*	*Clic4 Cxcl10 E2f7 E2f8 Foxm1 Lgals3 Nrp1 Plau Prdm1*

Only genes that were upregulated in at least two data sets are included.

noting that in this study microglial cells were treated for several hours to define early responses and this could be partially responsible for small changes in the pre-defined markers. Nonetheless, activated pathways and biological processes show similarities in microglial responses. One of most established markers of polarized microglia in glioma is Arginase 1 (*Arg1*). *Arg1* reduces levels of L-arginine that is essential for normal T-cell function. The increased Arg1 expression was associated with the immunosuppressive phenotype in microglia/macrophages in rat and mice glioma models (10, 15, 27). A recent study reported that inhibition of *Arg1* with the specific inhibitor in murine gliomas leads to the improved antitumor immune response and reduced tumor size (28). We report the increased *Arg1* expression in murine microglia exposed to human glioma GCM, suggesting the activation of immunesuppressive phenotype in these cells. Interestingly, *Arg1* expression is not significantly upregulated in published data sets of GAMs.

Nonetheless, global gene expression analysis revealed genes associated with immune response and immunosuppression (*Pmepa1*, *Ctla2a/b*, *Larc15a*, *Tgfbi*, and *Tgm2*). Activation of TGFβ signaling pathway has been corroborated by Western blot results and detection of the increased levels of phosphorylated Smad2 in microglia after treatment with GCM from LN18 and U87-MG cultures for 6 h. Signaling associated with TGFβ pathway and increased *Tgfbi* expression have been reported in microglia from GL261 gliomas (5). *Tgm2* expression is also induced by TGFβ pathway, participates in immune responses, and contributes to functions of monocytes/macrophages from migration to differentiation (29).

Interestingly, while GCM from all tested glioma cell lines induced the proinvasive activation of microglia, and in case of LN18 and U87MG increases in gene expression, GCM from U87-MG was particularly potent in induction of phagocytosis, morphological transformation, and tumor invasion support. All those cellular events are regulated in microglia by integrin signaling and mediated by activation of focal adhesion kinase/FAK-PI3K-Akt signaling. We have previously reported that tumor derived osteopontin, a ligand of integrins αvβ3, induces FAK-PI3K-Akt signaling in rat microglia (16). In fact, we found that U87-MG cells express and secrete larger quantities of osteopontin than other glioma cells (29), so it may explain the strongest effects of GCM from U87-MG cells on integrin signaling related events in microglia.

A Critical Assessment of Gene Expression Markers of Glioma-Induced Microglia Polarization

Classification of macrophage functions according to M1 and M2 phenotypes have been based on *in vitro* studies (30), but those phenotypes failed to be reproduced *in vivo*. It has been suggested that GAMs do not undergo an M2 polarization, but rather present a mixture of M1, and M2a,b,c-specific genes (5).

Our comparative analysis of GAM transcriptomics across different *in vivo* models—human, mouse, and rat, failed to reproduce consistent microglia phenotypes that could be classified according to previously reported gene signatures and showed remarkably low similarity between models. A mouse GL261 model is most commonly used in studies on glioma pathobiology, and thus, it is best characterized. However, our results show a stronger similarity of human GAMs gene expression profiles with transcriptomes of microglia infiltrating rat C6 gliomas, than murine GL261 gliomas (Figure S4 in Supplementary Material; **Table 2**). Both rat C6 and murine GL261 gliomas are cell line allografts that allow recapitulating the immune system response (31, 32), which might be limited in human xenograft models utilizing immunodeficient animals. C6 cell line produces tumors in outbred Wistars rat and has been reported to mimic best the gene expression changes of human glioma, among other rat cell lines (33). On the other hand, mouse GL261 cells form aggressive tumors in syngeneic C57BL/6 strains and present several molecular alterations that are commonly found in human gliomas: *p53* and *K-ras* mutations, MHCI, and MHCII downregulation (32).

The discrepancies and the low commonality in presented data sets might stem from different sources. The analyzed data sets were collected by different groups, using different methodologies (RNA-seq, microarrays) and different platforms for high-throughput transcriptome analysis, which could lead to an increased rate of false negatives and did not allow us to detect less pronounced effects. Even more important constrain which may hamper our analyses stems from methodological differences in GAM isolation, namely, FACS-based immunosorting versus immunomagnetic beads which may result in different degree of sample purity. Magnetic-activated cell sorting is thought to exert less shear stress on sorted cells than FACS. However, FACS offers a possibility to separate cells based on several markers simultaneously, allows for adjustments in gating strategy, and yields better purity.

Analyzed data sets were collected utilizing distinct markers: CD14 or CD11b or CD11bCD45$^{low/high}$ cells, which may result in different subpopulations with discrete functions. Unification of protocols, better separation of different subpopulations, or single-cell sequencing may bring more conclusive results.

Summarizing, despite of all difficulties we have identified two gene candidates for an universal GAM marker in experimental gliomas—*Tgm2* and *Gpnmb*, which should be further investigated. In addition, our functional analysis shows that pathways regulating the similar cell functions are upregulated across all models. It suggests that studies on universal mechanisms orchestrating an adaptation of the tumor-supporting phenotype by microglia and macrophages should be focused on functional groups rather than on individual genes.

ETHICS STATEMENT

The experiments were performed on glia cultures developed from mice pups. According to EU regulations acquiring tissues from laboratory animals does not require a permission from the Ethical committee.

AUTHOR CONTRIBUTIONS

KWalentynowicz and BK design the study, interpreted data, and wrote the manuscript. KWalentynowicz performed most of functional and microarray experiments. NO performed transcriptomic data analyses on public data sets. MP and KWojnicki performed invasion and SqPCR assays. KS performed microarray experiments. JM performed computational analyses. IC developed primary GBM cell cultures. All the authors wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01329/full#supplementary-material.

FIGURE S1 | Matrigel assay was performed to determine invasion of different human glioma cells in the presence of SV40 immortalized human microglia. Data are calculated as fold change in relation to basal invasion in the absence of microglia. Matrigel invasion data are calculated as means ± SD, $n = 3$ and were analyzed by one-sided paired sample *t*-test; $n \geq 3$. Differences at $p < 0.05$ were considered as significant (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$).

FIGURE S2 | Microarray gene expression validation. Five genes were selected for validation of the microarray gene expression analysis (*Tgm2, Cx3cr1, Tgfbi, Tmem37*, and *Socs2*). Expression is presented as delta Ct values in control, glioma-conditioned medium (GCM) LN18, and GCM U87-MG treated for 6 h. Validation was performed on separate experiments $n = 4$; **$p < 0.001$, *$p < 0.05$.

FIGURE S3 | Western blot analysis of total extracts from microglia polarized with glioma cells for active components of transforming growth factor beta signaling pathway revealed the increased levels of active, phosphorylated SMAD2 proteins accumulating at 3 and 6 h after glioma-conditioned medium (GCM) treatment. Immunoblots were stripped and re-probed first with an antibody recognizing total SMAD2 followed by detection of actin (used as a loading control).

FIGURE S4 | Correlation of gene expression patterns in various datasets. A heatmap shows Spearman's correlation coefficients between microglia activation (log2 fold changes) for samples collected from human, mouse, and rat samples.

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Early Microglia Activation Precedes Photoreceptor Degeneration in a Mouse Model of CNGB1-Linked Retinitis Pigmentosa

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Retinitis pigmentosa (RP) denotes a family of inherited blinding eye diseases characterized by progressive degeneration of rod and cone photoreceptors in the retina. In most cases, a rod-specific genetic defect results in early functional loss and degeneration of rods, which is followed by degeneration of cones and loss of daylight vision at later stages. Microglial cells, the immune cells of the central nervous system, are activated in retinas of RP patients and in several RP mouse models. However, it is still a matter of debate whether activated microglial cells may be responsible for the amplification of the typical degenerative processes. Here, we used *Cngb1*$^{-/-}$ mice, which represent a slow degenerative mouse model of RP, to investigate the extent of microglia activation in retinal degeneration. With a combination of FACS analysis, immunohistochemistry and gene expression analysis we established that microglia in the *Cngb1*$^{-/-}$ retina were already activated in an early, predegenerative stage of the disease. The evidence available so far suggests that early retinal microglia activation represents a first step in RP, which might initiate or accelerate photoreceptor degeneration.

Keywords: retinitis pigmentosa, retinal degeneration, cyclic nucleotide-gated channel, microglia, innate immune response

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INTRODUCTION

It is generally accepted that immune responses follow injury and damage to tissues and organs. Microglia are the resident immune cells within the brain and retina, commonly known as the macrophages of the central nervous system (CNS). In response to injury or inflammatory stimuli, the resting microglia can be rapidly activated to participate in pathological responses, including migration to the affected site, release of various inflammatory molecules, and clearing of cellular debris (1–3). Although microglia are essential for maintaining a healthy CNS, paradoxically they may undergo phenotypic changes to influence several neurodegenerative diseases and psychiatric disorders including Alzheimer's disease (AD), Parkinson's disease, and Rett syndrome (4). Moreover,

activation of microglia has also been detected in several retinal degenerative mouse models (5, 6) and in patients suffering from retinitis pigmentosa (RP) (7). RP describes a heterogeneous group of hereditary retinal degenerations with a world-wide prevalence of 1:4,000 (8). To date, more than 50 different genetic mutations have been detected, which cause non-syndromic RP (9). RP is characterized by an initial progressive degeneration of rods and followed by the loss of cones leading to severe visual impairment (8, 10). It should be noted that the disease severity, rate of disease progression, age of onset and clinical findings may differ significantly among patients based on the fact that RP represents a heterogeneous group of inherited retinal disorders (11). Typically, the earliest clinical symptom of RP is an initial night blindness caused by the dysfunctional rod system. Subsequent degeneration of cones leads to a gradual loss of the visual field, which initially impairs the periphery and spreads to the macula. The consequences include so-called "tunnel vision" and eventually complete blindness (10).

Here, we used the *Cngb1* knockout (*Cngb1*$^{-/-}$) mouse to study the activation of immune cells in a model of RP with slowly progressing photoreceptor degeneration. *Cngb1* encodes the B subunit of the cyclic nucleotide-gated channel in rod photoreceptors. *Cngb1*$^{-/-}$ mice show initial signs of rod degeneration including gliosis already between 14 and 21 days of age (12) while the peak of neuronal cell death occurs around 4 weeks of age (13). Even though the degenerative process begins already at this early age, the degeneration advances very slowly and shows a slower progression of the disease when compared with other RP mouse models like the rd1 mice (14). For RP a general feature is that cone photoreceptors deteriorate secondary to rods with a considerable slower progression rate (12). In the present study, we found that microglia in the *Cngb1*$^{-/-}$ retina showed already increased cell numbers and pronounced activation in 4-week-old mice. At this time point only a minor photoreceptor cell loss was detected. Our data suggest that *Cngb1*$^{-/-}$ microglia are potentially an early driving force, which substantially contributes to the retinal degeneration and long-term visual impairments found in RP.

MATERIALS AND METHODS

Animals

Cngb1$^{-/-}$ were generated by us (12). All mice used in the study were bred on a mixed genetic background of the 129Sv and C57BL/6N strain. Animals were housed under standard white light (200 lux, 12 h dark–light periods) with free access to food and water. Both male and female mice were used in equal shares. Age-matched wild-type mice were used as controls. Day of birth was considered as postnatal day 1 (P1). All procedures concerning animals were performed with permission of the local authority (Regierung von Oberbayern and RP Freiburg).

Optical Coherence Tomography (OCT) Analysis

For OCT examinations, mice received intraperitoneal injections of ketamin (0.1 mg/g) and xylazin (0.02 mg/g). Before the scanning procedure, Tropicamid eye drops were instilled into the eye for pupil dilation (Mydriadicum Stulln, Pharma Stulln GmbH, Stulln, Germany). Subsequently, hydroxylpropyl methylcellulose (Methocel 2%; OmniVision, Puchheim, Germany) was applied to keep the eyes moist. The examination was performed with an adapted Spectralis HRA + OCT system by Heidelberg Engineering (Dossenheim, Germany) in combination with optic lenses described previously (15). OCT scans were conducted using a 12 circular scan mode centered at the optic nerve head. This procedure allowed for measurements of the photoreceptor layer thickness at a comparable distance from the optic nerve head. In detail, outer nuclear layer (ONL) thickness was measured between the clearly visible outer limiting membrane and the outer plexiform layer (OPL). For statistical analysis, the mean ONL thickness was calculated from single values measured in the dorsal, temporal, nasal, and ventral region around the optic nerve.

Microarray Analysis

For microarray experiments, retinal tissue was obtained from mice of two different age groups (P12 and P28). For differential gene expression analysis of *Cngb1*$^{-/-}$ and wt animals, an Affymetrix platform was used according to the manufacturer's instructions as described before (16). In short, retinas were dissected, shock-frozen in liquid nitrogen and stored at −80°C until further use. RNA was extracted using RNeasy Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity were determined using NanoDrop2000 (Thermo Scientific®). Fragmented and labeled cRNA of three wild-type and three *Cngb1*$^{-/-}$ retinas was hybridized on Affymetrix Mouse Genome 430 2.0 Arrays, respectively. A probe-level summary was determined with the help of Affymetrix GeneChip Operating Software using the MAS5 algorithm. Raw data were normalized using the Array Assist Software 4.0 (Stratagene, La Jolla, CA, USA) in combination with the GC-robust multichip average algorithm. Significance was determined by a *t*-test without multiple testing correction (Array Assist software), selecting all transcripts with a minimum change in expression level of 1.5-fold together with a p–value <0.05.

Quantitative PCR

cDNA synthesis was performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's manual. PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using SYBR Select Master Mix (Applied Biosystems). For quantitative PCR, two technical replicates per gene were generated and normalized to the housekeeping gene aminolevulinic acid synthase. The following primer sets were used:

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Irf8	GCTGATCAAGGAACCTTGTG	CAGGCCTGCACTGGGCTG
Aif1/Iba-1	ATCAACAAGCAATTCCTCGATGA	CAGCATTCGCTTCAAGGACATA
C1qc	CCCAGTTGCCAGCCTCAAT	GGAGTCCATCATGCCCGTC
Cx3cr1	GAGTATGACGATTCTGCTGAGG	CAGACCGAACGTGAAGACGAG

Flow Cytometry

Cngb1$^{-/-}$ and wt mice were euthanized at P28 and perfused with phosphate-buffered saline. Retinas were removed and mechanically dissociated into single cell suspensions by pipetting. Dissociated cells were stained with live/dead dye (1:1,000, eBioscience) in PBS for 30 min at 4°C. In order to prevent unspecific binding to Fc receptors, their binding domains were blocked by unstained CD16/32 (1:250, 2.4G2, Becton Dickinson) in FACS-Buffer (2% FCS, 5 mM EDTA in PBS) for 20 min at 4°C. Cells were stained with CD11b (BV421, 1:300, M1/70, eBioscience), CD45 (APC-eF780, 1:200, 30-F11, eBioscience), F4/80 (PE; 1:200, BM8, eBioscience), CD44 (PE, 1:200 IM7, Becton Dickinson), and MHC class II (PE, 1:200, M5/114.15.2, eBioscience) in FACS-Buffer at 4°C for 20 min and analyzed using a FACSCanto II (Becton Dickinson). Viable cells were gated by forward and side scatter pattern. Data were acquired with FACSdiva software (Becton Dickinson). Postacquisition analysis was performed using FlowJo software (Tree Star, Inc.).

Retina Preparation and Immunohistochemistry

Retinas were dissected at P28 and further processed as described for immunohistology and whole mount preparation (17–19). Primary antibodies were added overnight at a dilution of 1:500 for Iba-1 (019-19741, WACO, Japan), 1:250 for Lamp 2 (ab13524, Abcam, Cambridge, UK), 1:200 for cleaved caspase-3 (9661, Cell Signaling Technology, Danvers, MA, USA) and 1:100 for Mhc class II (ab23990, Abcam), at 4°C. Secondary antibodies were added at the following dilution: Alexa Flour 488 1:500, Alexa Flour 555 1:500 and Alexa Fluor 568 1:500 for 2 h at room temperature. Nuclei were counterstained with DAPI. The examined area was determined microscopically by a TCS SP8 confocal scan microscope (Leica) or a conventional fluorescence microscope (Olympus BX-61).

Visual Cliff

The visual cliff behavior was analyzed in an open-top Plexiglas chamber. Half of the box protruded from the counter to provide a 3-foot depth. The box on the counter displayed a base with a checkerboard pattern and the box off the counter showed the base with the same checkerboard pattern, except for the 3 feet of depth. The mouse was placed on the dividing line between both halves of the chamber at the edge of the counter and was allowed to choose between the two sides. If the mouse stepped to the shallow side, time was scored as time spent on the "safe side." Each mouse performed this task twice for 10 min with a time window of 1 h between trials. The visual cliff behavior was averaged to generate mean percentage of time in which the mouse chose to stay at the shallow side ($n = 3$–5 mice per group).

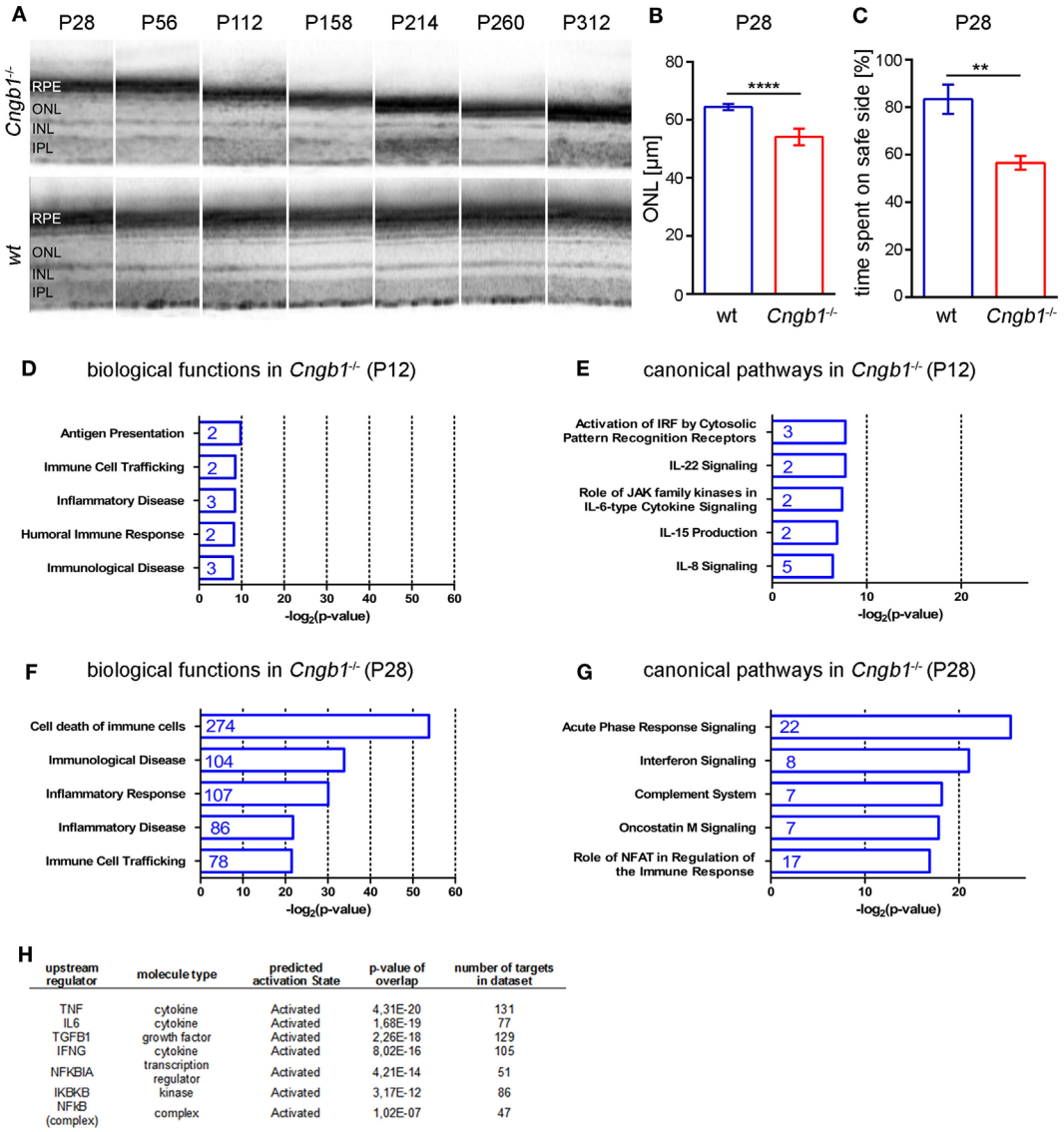
Statistical Analysis

All graphical data represent mean ± SEM. Sample sizes are provided in the figure legends. In order to test for significant differences, an unpaired *t*-test was applied. Differences were considered as significant when *p*-value <0.05.

RESULTS

At 28 days after birth (P28), minor (~15%) but significant retina degeneration was observed in *Cngb1*$^{-/-}$ mice (**Figures 1A,B**) (12) and more than 1,000 genes were dysregulated (>1.5-fold dysregulated, $p < 0.05$, STab.1) as seen from Affymetrix gene chip arrays. Already at this early time point *Cngb1*$^{-/-}$ mice displayed substantial visual impairment (**Figure 1C**). We were analyzing gene expression data with the help of Ingenuity Pathway Analysis software, to identify potential shifts in biological functions or in canonical pathways at early and predegenerative stages. Interestingly, activation of the immune system was already apparent 12 days after birth (P12) as indicated by upregulated genes that were involved in processes like antigen presentation, immune cell trafficking, immunological diseases, humoral immune response, and inflammatory disease (**Figures 1D,E**). At P28 many of the dysregulated genes were attributed to cell death, survival, or neurological diseases and also to pathways and signaling cascades that are assigned to immunological processes (**Figures 1F,G**; Table S1 in Supplementary Material). Particularly genes linked to inflammatory responses, inflammatory diseases and immune cell trafficking were significantly altered in *Cngb1*$^{-/-}$ retinas at P28 when compared to wt retinas. A detailed analysis of upstream regulators (URs), which are not directly altered in their expression level but are responsible for expression changes of their target genes, revealed the activation of diverse proinflammatory mediators like TNF, IL6, and NF-κB in *Cngb1*$^{-/-}$ retinas at P28 (**Figure 1H**).

The generated microarray data clearly suggested the presence of an activated immune system in *Cngb1*$^{-/-}$ retinas. That is why we focused on microglial cells, which represent the immune competent cells of the CNS and retina (5). Microglia tend to proliferate upon tissue destruction during neurodegeneration in order to clear the cellular debris and to restore tissue homeostasis (4). Thus, we first determined microglia cell numbers using flow cytometry (FACS), histological and qPCR approaches. For FACS analysis, we gated microglia as live CD45^{lo}CD11b^{+} cells (**Figures 2A,B**). Quantification of single cell suspensions prepared from *Cngb1*$^{-/-}$ and wt retinas 4 weeks after birth revealed significantly increased microglia cell numbers in *Cngb1*-deficient mice compared to age-matched wt (**Figure 2B**). CD45^{hi}CD11b^{+} cell numbers were not increased (**Figure 2B**). Immunofluorescence of Iba-1, a specific marker for microglia and macrophages, confirmed a strong elevation of this immune cell population in retinas of *Cngb1*$^{-/-}$ mice (**Figures 2C,D**). In addition to increased Iba-1^{+} cell numbers in degenerating retinas of *Cngb1*$^{-/-}$ mice, microglial cells also changed their localization. In wt retinas, microglia cells were mainly found in the inner plexiform layer (IPL) or OPL, while Iba-1-positive cells of *Cngb1*$^{-/-}$ retinas were additionally found in the ONL and in the photoreceptor layer close to the retinal epithelium (**Figure 2C**, asterisk; **Figures 3A,C**). Further analysis of wt and *Cngb1*$^{-/-}$ retina microarray data indicated that several microglia-specific genes like *Cx3cr1, Aif1, Irf8, C1qc* (20–22) were upregulated in the *Cngb1*$^{-/-}$ group (Table S1 in Supplementary Material). Subsequent RT-qPCR analysis of these microglia cell-specific genes confirmed their increased expression levels (**Figure 2E**). In summary, microglial cell numbers were strongly



H

upstream regulator	molecule type	predicted activation State	p-value of overlap	number of targets in dataset
TNF	cytokine	Activated	4,31E-20	131
IL6	cytokine	Activated	1,68E-19	77
TGFB1	growth factor	Activated	2,26E-18	129
IFNG	cytokine	Activated	8,02E-16	105
NFKBIA	transcription regulator	Activated	4,21E-14	51
IKBKB	kinase	Activated	3,17E-12	86
NFkB (complex)	complex	Activated	1,02E-07	47

FIGURE 1 | Activation of immunological pathways in *Cngb1*$^{-/-}$ retinas at 12 and 28 days after birth. **(A)** Representative optical coherence tomography (OCT) images from *Cngb1*$^{-/-}$ (upper panel) and wild-type retinas (lower panel), which display the slow progression (P28-P312) of outer nuclear layer (ONL) thinning over time. **(B)** Quantification of ONL thickness from OCT data at P28 (****$p < 0.0001$, $n = 6$ for each genotype). **(C)** Performance in the visual cliff test for the study of visual depth perception (**$p < 0.01$, $n = 3$ for each genotype). **(D–G)** Biological functions and canonical pathways were significantly altered in *Cngb1*$^{-/-}$ mice compared to age-matched controls. A high number of genes were related to the immune system or to immune responses. **(H)** The indicated upstream regulators for proinflammatory cytokines and the NF-κB pathway were predicted to have a significantly higher activation state in *Cngb1*$^{-/-}$ retinas when compared to age-matched wild-type controls ($p < 0.05$).

increased in *Cngb1*$^{-/-}$ mice at 4 weeks of age, which corresponds to an early, degenerative stage of the disease.

In response to disrupted tissue homeostasis, microglial cells get activated and change their morphology together with the expression of surface markers (4). In *Cngb1*$^{-/-}$ retinas, microglia showed a transition from a resting to an activated state (**Figure 3A**). The cells underwent morphological changes to take on an amoeboid shape with fewer branches compared to the resting state phenotype in wt retinas (23, 24). As specialized phagocytes, one of the functions microglia have is to remove debris of dying or dead cells (25). In mice, CD44 is a competent receptor for phagocytosis in macrophages (26) and an increase of CD44 expression was detected in the initial microarray data analysis (STab.1). Subsequent FACS expression analysis of CD45^{lo}CD11b^{+} cells could link CD44 to microglia, as the number of CD45^{lo}CD11b^{+}CD44^{+} cells as well as the expression levels of CD44 in microglia in *Cngb1*$^{-/-}$ retinas were elevated (**Figure 3B**). Active phagocytosis of microglial cells can also be monitored *in situ* by immunohistological staining of lysosome-associated membrane protein (lamp)-2 (19). In P28 *Cngb1*$^{-/-}$ retinas, we

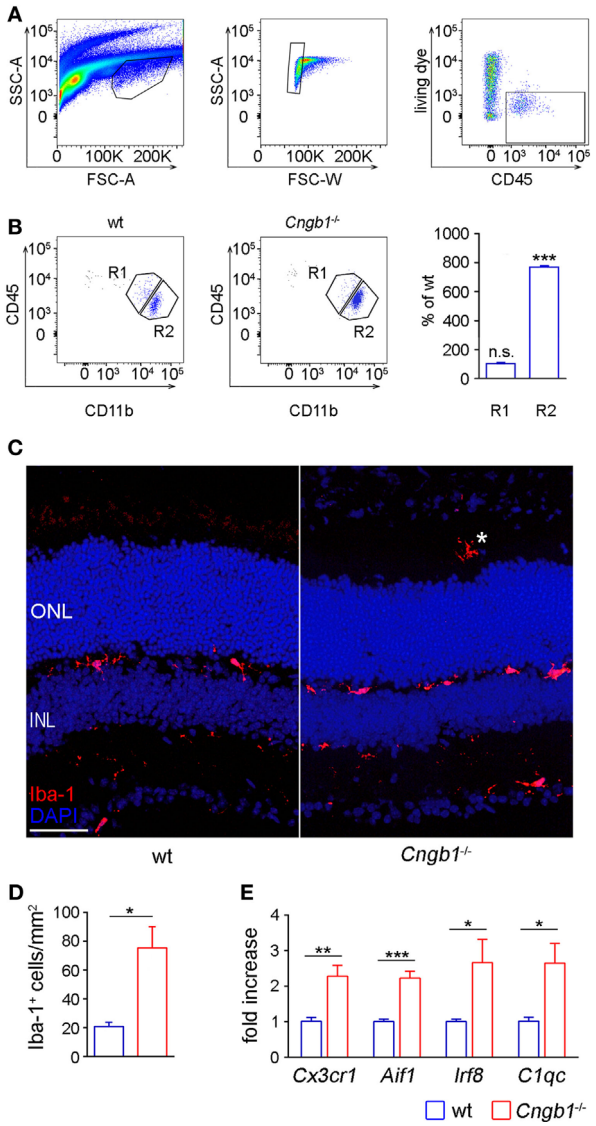


FIGURE 2 | Strong increase of retinal microglia cells in *Cngb1*$^{-/-}$ retinas 28 days after birth. **(A)** Gating strategy for identifying microglia cells in retina lysates by size (left panel), single cells (middle panel), and living CD45^{+} cells (right panel) expression. **(B)** Representative gating and quantification of CD11b^{+}CD45^{low} retinal microglia (R2) and CD11b^{+}CD45^{hi} cells (R1) from wild-type or *Cngb1*$^{-/-}$ retinas. (Statistical significance was determined vs. percentage of wild-type cell counts, ns = non significant; ***$p < 0.001$, $n = 3$ for each genotype.) **(C)** Immunofluorescence of Iba-1 (red) in the retina of *Cngb1*$^{-/-}$ and age-matched wt demonstrating migration of microglia into the photoreceptor layer of *Cngb1*$^{-/-}$ mice (asterisk). Nuclei were counterstained with DAPI. ONL, outer nuclear layer, INL, inner nuclear layer, scale bar 50 µm. **(D)** Quantification of Iba-1-positive cells in *Cngb1*$^{-/-}$ mice compared to wt animals (*$p < 0.05$, $n = 4$ for each genotype). **(E)** Gene expression of microglia-specific genes in *Cngb1*$^{-/-}$ and wt retinas. Significant increase in the expression of *Cx3cr1, Aif1*, *Irf8*, and *C1qc* in retinas of *Cngb1*-deficient mice (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 4$ for each genotype).

detected Lamp-2-positive microglia particularly in the ONL and photoreceptor layer (**Figure 3C**). Increased MHC class II expression, which indicates microglial activation, was further observed by FACS and immunohistochemistry in *Cngb1*$^{-/-}$ retinas when compared to wt retinas (**Figure 3D**), while the expression levels of the macrophage marker F4/80 remained unchanged in both genotypes (**Figure 3B**). Although the neurodegenerative process became evident at P28, microglia were activated already at P12 (**Figures 4A,C–E**). At this time point no apoptotic cells as indicated by the absence of positive signals for cleaved caspase 3 were present (**Figures 4B,F**).

The Ingenuity UR analysis indicated increased activity of diverse proinflammatory signaling cascades (**Figure 1H**). One of these cascades was the NF-κB pathway, which can be induced by a variety of signals to finally induce a specific pattern of transcription. In this classical pathway, activated IKK-β, which is part of an IKK-α–IKK-β–IKK-γ complex, phosphorylates the inhibitory subunits IkB-α, IkB-β, or IkB-ϵ, leading to their proteasomal degradation. As a result, NF-κB homodimers and heterodimers, mainly composed of RelA, RelC, and p50, accumulate in the nucleus (27). Here, we confirmed the presence of an activated NF-κB-signaling pathway in microglia by the immunofluorescent detection of phosphorylated IκB colocalized with Iba-1-positive microglia, which were misplaced in the outer segment of *Cngb1*$^{-/-}$ retinas (**Figure 5**).

DISCUSSION

Our present results link retinal degeneration to immune system activation, and here, more precisely, to the activation of microglia. In *Cngb1*$^{-/-}$ mice, neuronal cell death does not start before postnatal day 15 (P15) (12). Already before this early stage of degeneration, retinal gene expression analysis at P12 indicated an immune response in biological and canonical pathways. These data clearly indicated that activation of the immune system starts prior to the actual retinal degenerative process in *Cngb1*$^{-/-}$ mice. Between P21 and P28 retinal degeneration reaches its maximum (12). We determined activation of the immune system by gene expression pathway analyses and immunohistochemical detection. At this early disease stage, microglia had already migrated entirely through the various layers of the *Cngb1*$^{-/-}$ retina toward the photoreceptors. Microglial cells are the local immune cells of the CNS and normally reside at the IPL/OPL of the retina (5, 28). Upon activation, microglia migrate toward the injury site, change their morphology from ramified cells to amoeboid phagocytes and start expressing several surface markers including F4/80, MHCII, and complement receptor 3 (CD11b/18, Ox42) (1, 5, 29). Our findings suggest that microglial activation occurs before the onset of neurodegeneration. This early microglia activation might be responsible for the observed high CD44 representation in *Cngb1*$^{-/-}$ retinas. CD44 is implicated in the pathogenesis of inflammation and contributes to the recruitment of inflammatory cells as well as to increased phagocytosis (26, 30, 31). Increased relative expression of the cell surface adhesion receptor CD44 seems to be a very general feature of retinal degeneration considering that it was also present in rd10 mice (32). Previous work using the rd10 mouse model of RP had already suggested a contribution of microglia in retinal degeneration (33). In this mouse model activated microglia infiltrate into the photoreceptor layer and contribute actively to photoreceptor demise *via* the

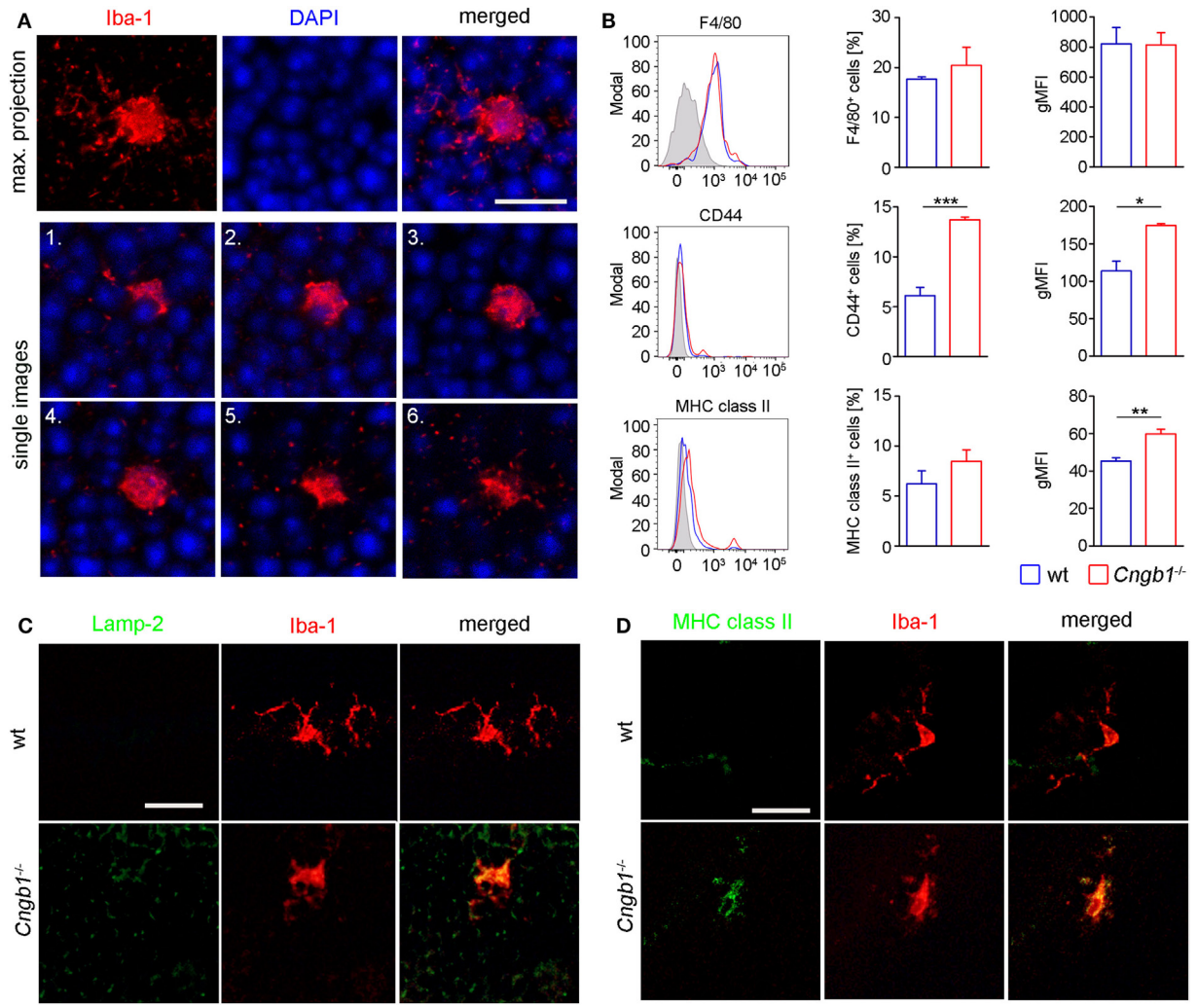


FIGURE 3 | Amoeboid microglia morphology in *Cngb1*$^{-/-}$ is accompanied by the expression of activation markers. **(A)** Whole mount images of Iba-1-stained microglia in the outer nuclear layer (ONL) of *Cngb1*$^{-/-}$ mice induced morphological changes from a resting to an amoeboid phenotype at P28. Nuclei were counterstained with DAPI. Upper panel: maximum projection. Lower two panels: single images with an increment of 1.3 µm. Scale bar. 10 µm. **(B)** Flow cytometric analysis of retinal microglia for the expression of F4/80, CD44, and MHC class II (left panel). Quantification of the numbers (middle panel) and geometric mean fluorescent intensities (gMFI, right panel) of F4/80, CD44, and MHC class II are depicted. Results were obtained from two independent experiments with at least three replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$ for each genotype, blue line = wt, red line = *Cngb1*$^{-/-}$, grey = isotype control). **(C)** Misplaced microglia in the ONL in *Cngb1*$^{-/-}$ coexpressed activation marker Lamp-2. Scale bar 25 µm. **(D)** Confirmation of MHC II expression by costaining of Iba-1 (red) and MHC class II (green) in wt and *Cngb1*$^{-/-}$ retinas. Scale bar 25 µm.

phagocytotic clearance of viable photoreceptors and the secretion of proinflammatory cytokines that potentiate photoreceptor apoptosis (33, 34). It still remained unclear whether microglial activation was responsible for further photoreceptor cell death. Even though, genetic depletion of microglia slowed down the degenerative process in rd10 mice (33). In follow-up experiments, it would be interesting to investigate whether microglial cells are actually the main detrimental force in *Cngb1*-deficient retinas. This could either be achieved by allowing CX3CR1^{+} retinal microglia to express diphtheria toxin and be specifically ablated upon tamoxifen administration (33, 35) or by pharmacological ablation using the CSF1R inhibitor (36).

Our results indicate that microglia activation is an important step in the degenerative process of rods in RP. The intriguing question however is whether microglia get activated during a predegenerative state or whether signals from a small number of degenerating cells is sufficient to initiate the activation of microglia before the actual "degeneration peak." In the rd10 retina activated microglia infiltrate the ONL at P16. Since photoreceptor apoptosis started only at P19 microglia activation preceded the initiation of photoreceptor apoptosis (37). Comparable findings of microglia proliferation and activation at early time points were also described in rd1 and rd10 mice, which represent further mouse models of RP (37–39). Both RD models are induced by a

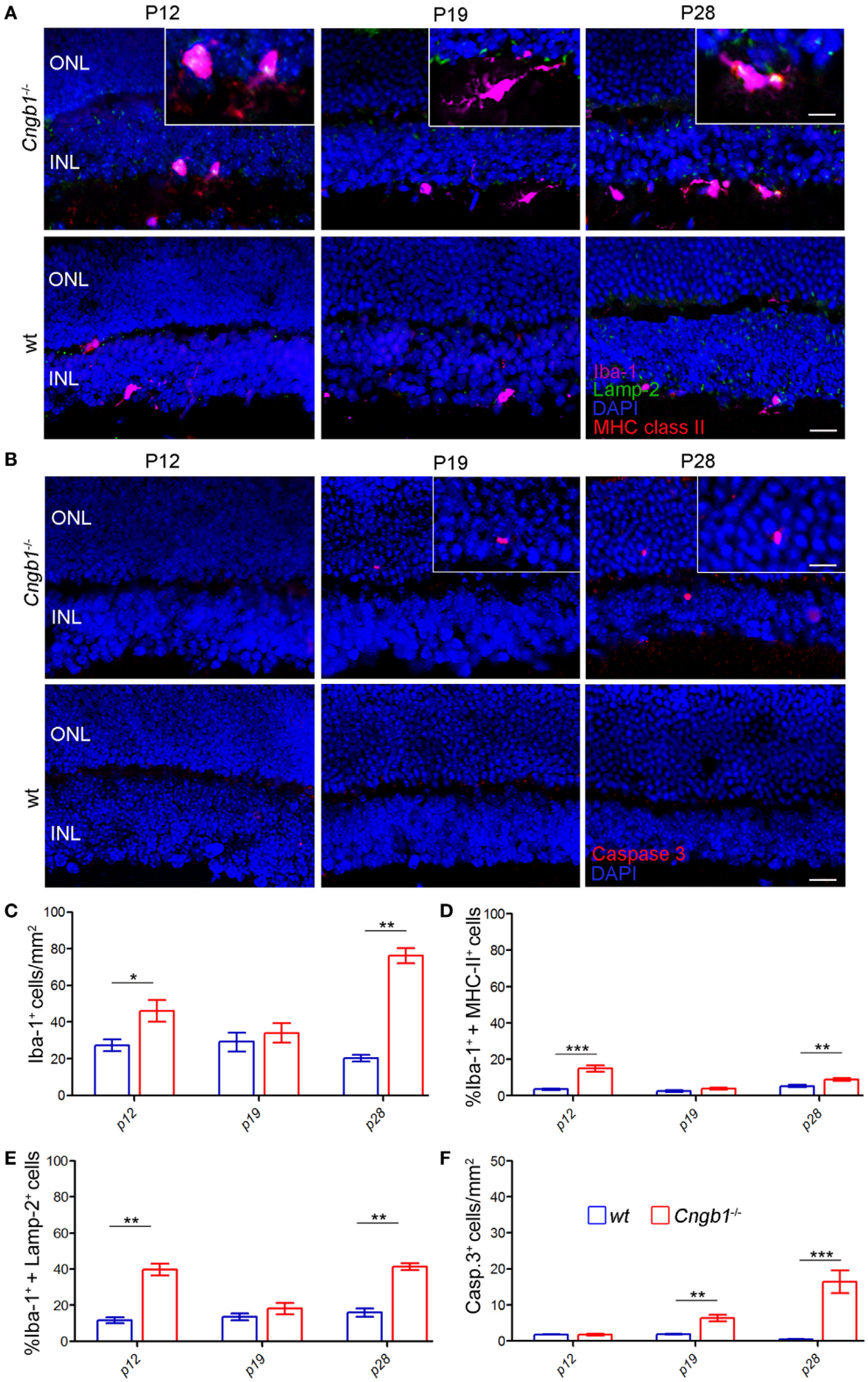


FIGURE 4 | Time course of early photoreceptor apoptosis and microglia activation. **(A)** Costaining of Iba-1-positive microglia (pink) in the ONL and INL of *Cngb1*$^{-/-}$ and wt mice at P12, P19 and P28 with the activation marker Lamp-2 (green) and MHC class II (red). **(B)** Immunofluorescence of cleaved caspase 3 (red) in *Cngb1*$^{-/-}$ and wt mice. Nuclei were counterstained with DAPI (blue). **(C)** Quantification of Iba-1-positive cells and the percentage of Iba-1^{+} MHC class II– **(D)** or Iba-1^{+} Lamp-2-positive cells **(E)** in *Cngb1*$^{-/-}$ mice compared to wt animals. **(F)** Cleaved caspase 3-positive cells in *Cngb1*$^{-/-}$ and wt mice at indicated time points. ONL, outer nuclear layer, INL, inner nuclear layer, scale bar 20 µm; insert 10 µm (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 5$ for each genotype).

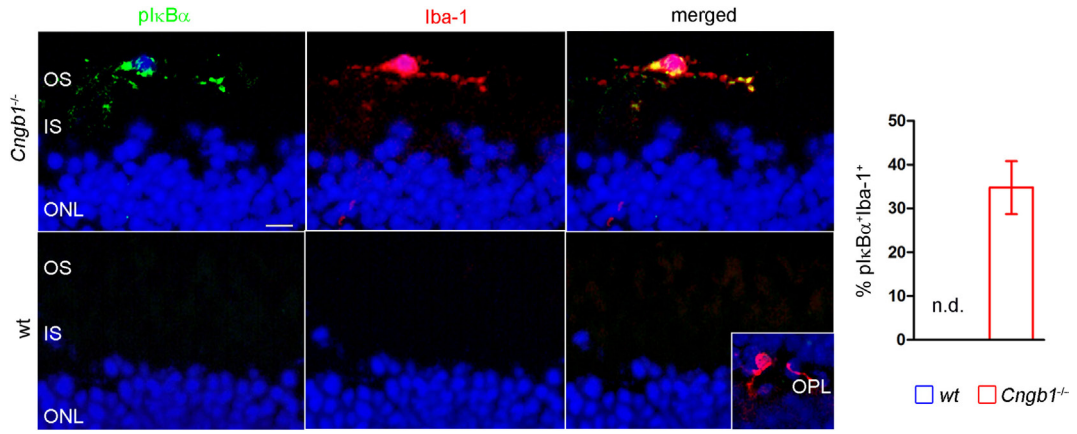


FIGURE 5 | Activation of proinflammatory signaling in *Cngb1*$^{-/-}$ mice. Immunofluorescence of Iba-1 (red) and pIκBα (green) in *Cngb1*$^{-/-}$ and wt mice at P28. Quantification of double-positive cells in wt and KO revealed exclusive presence of active NF-κB-signaling in *Cngb1*$^{-/-}$ mice ($n = 3$ for each genotype). Insert: Iba-1-positive resting microglia in the OPL. Nuclei were counterstained with DAPI. ONL, outer nuclear layer, IS, inner segment, OS, outer segment, OPL outer plexiform layer, scale bar 20 μm, nd = not detectable.

mutation in the rod photoreceptor-specific Pde6b gene (40, 41). In fact, retinal architecture in rd10 mice displayed alterations from as early as P5, which is at least 13 days before photoreceptor loss (39). These alterations included increased proliferation of microglia within the retina, which ultimately led to increased numbers of activated microglia. At the same time there was a significant decrease of glutamine synthetase in Müller glia followed by an increase in glial fibrillary acidic protein immunofluorescence, which is expressed in Müller glia and astrocytes (39). A similar activation of astrocytes might be present in *Cngb1*-deficient retinas when one considers the abundant LAMP-2 labeling outside microglial cells. To what extent this reactive gliosis contributes to photoreceptor degeneration in both mouse models is not clear. The observed microglia activation can play a critical role in neuroinflammation and impose subsequent damage with progressive photoreceptor loss (42). In contrast, microglia might also be beneficial during retinal degeneration. This assumption is based on studies showing that microglia-derived trophic support protects photoreceptors *in vivo* under stressful conditions (43). Our data suggest that resident microglia and not monocyte-derived macrophages are mainly involved in the neurodegenerative process. Both cell populations are phenotypically distinguishable with a unique microglial CD45^{lo} CD11c^{lo} F4/80^{lo} I-A/I-E^{lo} profile and a monocyte-derived macrophage CD45^{hi}CD11b^{hi} signature (44). However, it has also been shown that activated retinal microglia upregulate CD45 (45) and that differentiation of monocytes into macrophages may be associated with downregulation of CD45 sometimes to levels that make the two cell populations indistinguishable (46). The small CD45^{hi} CD11b^{-} cell population found in *Cngb1*-wt and *Cngb1*-ko retinas presumably represents circulating retina-specific T cells (47), which have been reported to protect against spontaneous organ-specific autoimmunity (48). At the molecular level, inflammation is often regulated by numerous molecules and factors, including the transcription factor NF-κB (49). The activation of NF-κB in microglia, as seen in our present RP mouse model, is often associated with the release of reactive oxygen species and proinflammatory cytokines (such as IL-1β, interferon-γ, and TNF-α) that can cause secondary neurotoxicity and neuronal cell death including the degeneration of photoreceptors (50). Dying photoreceptor cells, in turn, induce NF-κB in microglial cells and thereby further their activation (51). Detrimental NF-κB-signaling in microglia has a key role in several degenerative processes of the CNS as documented for aging including AD (52), amyotrophic lateral sclerosis (53), and multiple sclerosis (54). When mice and rats express mutant rhodopsin, they experience photoreceptor cell death and, much as humans, develop the clinical signs of autosomal dominant retinitis pigmentosa (ADRP). During the progression of ADRP, microglia get activated and display heightened NF-κB-signaling (55). Increased expression of NF-κB protein and NF-κB DNA-binding activity in microglia of the retina has also been reported during photoreceptor degeneration of *rd* mice. In this model, the neurotoxic role of microglial NF-κB activation in photoreceptor apoptosis was mediated by increased TNF-α production in microglial cells (56). Several studies have also indicated that NF-κB activation leads to enhanced IL-1β secretion by microglia, which makes them contribute to rod degeneration in RP by potentiating apoptosis (33).

In terms of therapy, targeting microglia may reduce the production of several proinflammatory mediators and may therefore result in broader therapeutic effects than inhibition of single cytokines. However, chemical or genetic depletion of microglia would provide an approach with only short-term beneficial effects since microglia has been shown to repopulate once the treatment ends (35, 36). Particular attention should be paid to unwanted depletion or damage to other cells like optic nerve oligodendrocyte precursor cells. As an example, secondary to microglia depletion by the CSF-1R inhibitor BLZ945, oligodendrocyte precursor cells are reduced in early, postnatal mouse brains (57). As recently described, tamoxifen, a selective estrogen receptor modulator approved for the treatment of breast cancer

and previously linked to a low incidence of retinal toxicity, was unexpectedly found to exert marked protective effects against photoreceptor degeneration. Tamoxifen treatment decreased retinal microglia activation in a genetic (*Pde6b*^{rd10}) model of RP and limited the production of inflammatory cytokines and as a consequence reduced microglial-mediated toxicity to photoreceptors (58). Minocycline, a semi-synthetic tetracycline derivative, prevents NF-κB activation by blockade of Toll-like receptor signaling and counteracts microglial release of TNF-α and IL-1β. This is probably why there are also good indications that minocycline is effective in dampening microglial neurotoxicity and to prevent photoreceptor apoptosis (37, 59). Like minocycline, sulforaphane, a naturally occurring isothiocyanate, also inhibits the proteolytic cleavage of NF-κB and inhibits light-induced photoreceptor apoptosis (60). In a similar manner, polysaccharides were effective in preserving photoreceptors against degeneration in rd10 mice partly through inhibition of NF-κB (61).

In conclusion, both strategies, inhibiting microglial activation and/or inhibition of NF-κB-signaling, can provide useful approaches to prevent retinal degeneration in RP.

ETHICS STATEMENT

All procedures concerning animals were performed with permission of the local authority (Regierung von Oberbayern and RP Freiburg).

AUTHOR CONTRIBUTIONS

TB, TG, and SM designed research. TB, TG, MK, LA, CS, MBo SP, MP, MBu, MBi, SM, and JW performed experiments, analyzed, and interpreted the data. TG and MK designed the figures. TG, TB, and SM wrote the manuscript. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

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Alpha-1 Antitrypsin Attenuates M1 Microglia-Mediated Neuroinflammation in Retinal Degeneration

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Neurodegenerative diseases are a set of disorders characterized by progressive neuronal death and are associated with microglia-mediated neuroinflammation. Recently, neuroinflammation is proposed as a promising therapeutic target for many neurodegenerative diseases. Alpha-1 antitrypsin (AAT) is recognized as a novel immunomodulatory agent in autoimmune diseases and transplantation, however, its impact on neuroinflammation and neurodegeneration remains unknown. This study aims to explore the effects of AAT on microglia-mediated neuroinflammation and retinal degeneration in rd1 mouse model. We found reduced expression of AAT in rd1 retina, and AAT supplement exhibited certain protective effect on retinal degeneration, presenting with increased amount of photoreceptor nuclei, and amplified wave amplitudes in electroretinogram analysis. Of note, AAT shifted microglia phenotype from pro-inflammatory M1 (CD16/CD32$^+$, iNOS$^+$) to anti-inflammatory M2 (CD206$^+$, Arg1$^+$) both *in vivo* and *in vitro*, underscoring the concept of immunomodulation on microglia polarization by AAT during neurodegeneration. Furthermore, AAT suppressed the activation of STAT1, promoted the expression of IRF4 while inhibited IRF8 expression, indicating the involvement of these signaling pathways in AAT immunomodulation. Collectively, our data provided evidence for a novel protective role of AAT through immunomodulation on microglia polarization. Attenuating neuroinflammation by AAT may be beneficial to retard neurodegeneration in rd1 mice.

Keywords: alpha-1 antitrypsin, immunomodulation, microglia polarization, rd1 mice, neuroinflammation

INTRODUCTION

Neurodegenerative diseases are a heterogeneous group of disorders characterized by progressive loss of specific neurons and association with neuroinflammation. Retinitis pigmentosa (RP), one of the most common inherited retinal degeneration, is a major cause of incurable vision loss (1). It is a highly complex neurodegenerative disease defined by progressive degeneration of photoreceptors or retinal pigment epithelium (RPE) caused by various mutations (2). So far, more than 200 mutant genes have been identified (3, 4) and gene therapy therefore faces the challenge of genetic heterogeneity,

Abbreviations: AAT, alpha-1 antitrypsin; RP, retinitis pigmentosa; RPE, retinal pigment epithelium; ERG, electroretinogram; OCT, optical coherence tomography; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; CNS, central nervous system; IRF, interferon-regulatory factor.

which makes mutation-independent approaches highly desirable (5). Drugs that target on the broad biological processes which are common across various mutations in RP may show some efficacy in this complicated disorder (6).

Neuroinflammation is now recognized as a hallmark of many chronic neurodegenerative disorders and attenuating neuroinflammation has been proved to be a potential therapeutic strategy for these diseases (7, 8). It has been reported in RP patients with a substantial number of inflammatory cells found in the vitreous cavity and significantly elevated expression of a variety of pro-inflammatory cytokines and chemokines (9), highlighting that inflammation was implicated in retinal degeneration. Growing evidence suggested that microglia, the tissue-resident immune cells, would initiate immune responses with a chronic inflammation sustained in RP (10). The onset of microglia-mediated inflammation is identified as a common feature of RP (11).

Alpha-1 antitrypsin (AAT), an endogenous serine protease inhibitor for neutrophil elastase (NE), is emerging as a novel immunomodulatory agent to intervene immune diseases and transplantation (12). It is mainly produced by hepatocytes and maintains homeostasis with normal serum levels of 2–5 mg/mL (13). Patients with inherited AAT deficiency are predisposed to the development of lung diseases due to excessive destructive NE (14). And the AAT augmentation is well acknowledged in clinic for the treatment of these individuals (15). Interestingly, it was reported that a patient with RP was identified heterozygous for AAT deficiency (16). It is still unknown if there is a relationship between AAT deficiency and RP, and whether AAT augmentation is suitable for RP patients. Actually, besides its ability for elastase degradation, AAT was recently reported to suppress adverse immune responses under various conditions. For example, AAT application could avert type 1 diabetes and prolong the survival of islet allograft in NOD mice (12, 17). AAT administration reduced the susceptibility of islets to inflammation (18). These findings proposed AAT as a novel alternate for immunosuppression. The microglia-mediated neuroinflammation is an important pathological process in neurodegenerative diseases and immune modulation targeting on microglia is a promising treatment for these diseases. Therefore, we proposed that AAT may have the potential to attenuate microglia-mediated neuroinflammation and retinal degeneration.

In the present study, the impact of AAT immunotherapy on retinal inflammation and neurodegeneration was examined in rd1 mice, a typical mouse model of RP, as well as in cell culture. The results showed that AAT suppressed microglia-mediated neuroinflammtion and protected the degenerative retina. Our data proved that AAT may be beneficial to retinal degeneration by shifting retinal microglia from proinflammatory M1 to anti-inflammatory M2 phenotype.

MATERIALS AND METHODS

Rd1 Mice and AAT Treatment

Rd1 (FVB/N) mice were purchased from Beijing Vital River Laboratory Animal Technology, Co., Beijing, China and were kept in a specific pathogen-free facility in Animal Laboratories of Zhongshan Ophthalmic Center. This study was approved by the animal experimental ethics committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (authorized number: 2014-039). All the experiments were carried out in accordance with the approved guidelines of Animal Care and Use Committee of Zhongshan Ophthalmic Center and the Association Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Rd1 mice and C57BL/6J mice at P4, P10, P14, and P20 were sacrificed for detecting AAT expression in retina. For treatment, rd1 mice received intraperitoneal injections of AAT (soluted in PBS 80 mg/kg, Sigma Chemical Co., St. Louis) every other day from P4 to P14. Mice were sacrificed at P16 and eyes were enucleated for further investigation, normal C57 mice of the same age were used as the control.

Primary Microglia and BV2 Cell Line Culture

Primary microglia were isolated and cultured as described previously (19). In brief, retinae were collected from 3-day-old Sprague–Dawley rats and crashed drastically into single cell suspensions. The cells were then resuspended in DMEM/F-12 medium containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, and were seeded at a density of 1×10^6 cells/mL. The culture medium was changed 24 h later and twice a week thereafter. 2 weeks later, after shaking the flasks at 200 rpm for 1 h, the suspensions were collected and centrifuged at $300 \times g$ for 8 min at 4°C. The cells were harvested and identified as microglia using IBA1 staining. The BV2 murine microglial cell line was purchased from Kunming Institute of Zoology, Chinese Academy of Sciences, China. The microglia cells were pre-treated with AAT (1 mg/mL dissolved in PBS, A6105, Sigma) or PBS for 2 h and then stimulated with hydrogen peroxide (200 μM). Eighteen hours later, the cells were harvested for further analysis.

Electroretinogram (ERG) Recordings

In vivo ERG were performed on rd1 mice treated with AAT or PBS ($n = 9$ for each group) and C57 mice of the same age ($n = 6$). After dark adaptation overnight, mice were anesthetized with pentobarbital sodium diluted in saline. The pupils were dilated with 1% tropicamide and 2.5% phenylephrine and the corneas were anesthetized with 0.5% tetracaine HCl eye drops. ERGs were recorded with gold plated wire loop electrodes contacting the cornea surface as the active electrodes. Stainless steel needle electrodes were inserted into the skin near the eye and into the tail serving as reference and ground leads respectively. The mice were exposed to full-field scotopic flashes of 1.3 ms duration presented by a Ganzfeld (Roland Consult, Germany) with the intensities of 3.0 and 10.0 log cd•s/m^2. For each intensity, five responses were averaged to the luminance of flash stimuli. Amplitudes of the major ERG components (a- and b-wave) were measured (RETIsystem software) using automated and manual modes.

Imaging Mouse Retina by Optical Coherence Tomography (OCT)

Optical coherence tomography examination with SPECTRALIS-OCT (Heidelberg, Germany) was performed to detect the retinal

structure in rd1 mice treated with AAT or PBS ($n = 9$ for each group) and normal C57 mice ($n = 6$). Briefly, the mice were anesthetized with pentobarbital sodium diluted in saline and the pupils were dilated with 1% tropicamide and 2.5% phenylephrine. Each scan was performed for at least two times, with realignment each time. Both the nasal and temporal retinae within 6×3 mm^2 area adjacent to optic nerve disk were scanned, consisted of 25 scan slices to form 3D acquisition. From the 3D imaging, average retinal thickness was measured within a circle area, with 1.5 mm in radius, centering at 3 mm away from the optic nerve disk.

Hematoxylin & Eosin Staining

Eyes were fixed in formalin overnight, embedded in paraffin, and were cut into 3 μm vertical slices. Sections were washed in deionized water for 5 min and incubated with hematoxylin buffer for 10 min at room temperature. Then these sections were rinsed in deionized water and dipped in 1% eosin solution for 15 s. After rehydrated in alcohol gradients, slices were washed again and mounted. Histological analyses of retinal tissues were observed under microscope (Leica DM4000, Germany). The amount of cell nuclei in outer nuclear layer (ONL) was counted and analyzed. Images were processed and analyzed with Image J software (Public Domain, imagej.nih.gov/ij/).

Immunofluorescence Staining on Retinal Cryosection and Whole Mounts

Eyes were enucleated and fixed in 4% paraformaldehyde for 60 min. For cryosection, eyes were embedded in O.C.T. compound (Tissue-Tek) with adjustment of the rim of corneal limbus in vertical direction. The frozen sample was then sliced transversely (8 μm) with a cryostat at −20°C and the cross-sections throughout optic nerve were used for staining and analysis. For retinal whole mounts, the retinae were dissected out. Both cryosections and retinal whole mounts were blocked with 0.5% Triton X-100/5% BSA for 2 h at room temperature and were incubated with primary antibodies overnight at 4°C. After washing with PBS, the slices were incubated with secondary antibodies for 1 h and counterstained with DAPI (Invitrogen) for 5 min before mounted. The primary antibodies included anti-AAT antibody (ab166610, Abcam, MA), anti-Brn3a antibody (ab81213, Abcam), anti-GFAP antibody (ab7260, Abcam), anti-CD11b antibody (ab8878, Abcam), anti-CD68 antibody (ab53444, Abcam), anti-IBA1 antibody (019-19471, Wako; ab15690, Abcam), anti-CD16/32 antibody (553141, BD Biosciences), anti-CD206 antibody (AF2535, R&D Systems), anti-iNOS antibody, anti-Arg1 antibody, and anti-Rhodopsin antibody (sc-7271, sc-18355, sc-57432, Santa Cruz, MA, USA). Antibodies used in this experiment were summarized in Table S1 in Supplementary Material. TUNEL staining (*In Situ* Cell Death Detection Kit, Fluorescein; Roche, IN, USA) was performed according to the manufacturer's instructions. The images were obtained using Zeiss Axiophot fluorescent microscope and LSCM (LSM710, Carl Zeiss).

Western Blotting

Retinal and cellular protein were harvested and homogenized in lysis buffer (RIPA, Biocolors, Shanghai, China) containing protease and phosphatase inhibitor mini tablets (Thermo Fisher Scientific, No. 88668; USA). The protein concentration was determined by bicinchoninic acid protein assay. Equal amount of protein was used and western blotting was performed as previously described (20). Primary antibodies included anti-AAT antibody (ab166610, Abcam, MA), anti-STAT1 antibody, anti-pSTAT1 antibody (14994S, 7649S, CST), anti-IRF4 antibody (PA5-21144, Thermo Fisher Scientific, USA), anti-IRF8 antibody, anti β-actin antibody (ab28696, ab28696, Abcam, MA, USA), anti-iNOS antibody, and anti-Arg1 antibody (sc-7271, sc-18355, Santa Cruz, CA, USA). The gray intensity of protein blots was measured using Image J software (US National Institutes of Health).

Quantitative PCR Analysis

The mRNA levels of AAT were detected by real time PCR. The total RNA of retinae were extracted with TRIzol (Invitrogen) and converted into first-strand cDNA using random hexamer primers and the Reverse Transcriptase Superscript II Kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in a total volume of 20 μL containing 2 μL of cDNA, 10 μL of 2 × SYBR Premix Ex Taq, 7 μL ddH_2O, and 10 μmol/L of the primer pairs. The sequence of the used primers was: AAT forward: 5′-TCCCATGAGATCGCTACAAAC-3′; reverse: 5′-TGATAATGGTTCTTGGCCTCT-3′; GAPDH forward: 5′-GCCAAGGCTGTGGGCAAGGT-3′; reverse: 5′-TCTCCAGGCGGCACGTCAGA-3′. The PCR amplification protocols consisted of 95°C for 30 s and up to 40 cycles of 95°C for 5 s and 60°C for 34 s according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Version 6.0, La Jolla, CA, USA). For immunofluorescence on retinal whole mounts, three images were captured in the center, mid-periphery, and periphery areas of each retina respectively. Six retinae from six mice were used in each group for analysis. Representative images were shown in the according Figures. For immunofluorescence, Hematoxylin & eosin, and TUNEL staining on retinal section, at least three sections of each retina and three retinae of each group were used. In every section, three images were captured in the center, mid-periphery, and periphery area, respectively. In experiments including qPCR and western blot, samples were collected from three retinae of three individual mice. All *in vitro* experiments were performed in triplicate and repeated independently for at least three times. Unpaired student *t*-test was used to compare the means between two groups. Data were presented as mean ± SEM and P values <0.05 were considered statistically significant.

RESULTS

The Expression of AAT Was Reduced in the Degenerative Retinae of rd1 Mice

Although AAT is mainly produced by hepatocytes, it could also derive from macrophages, monocytes, and other cells (21, 22). We first detected the expression level of endogenic AAT in rd1 mice retinae. Strikingly, the RNA (**Figure 1A**) and protein (**Figure 1B**)

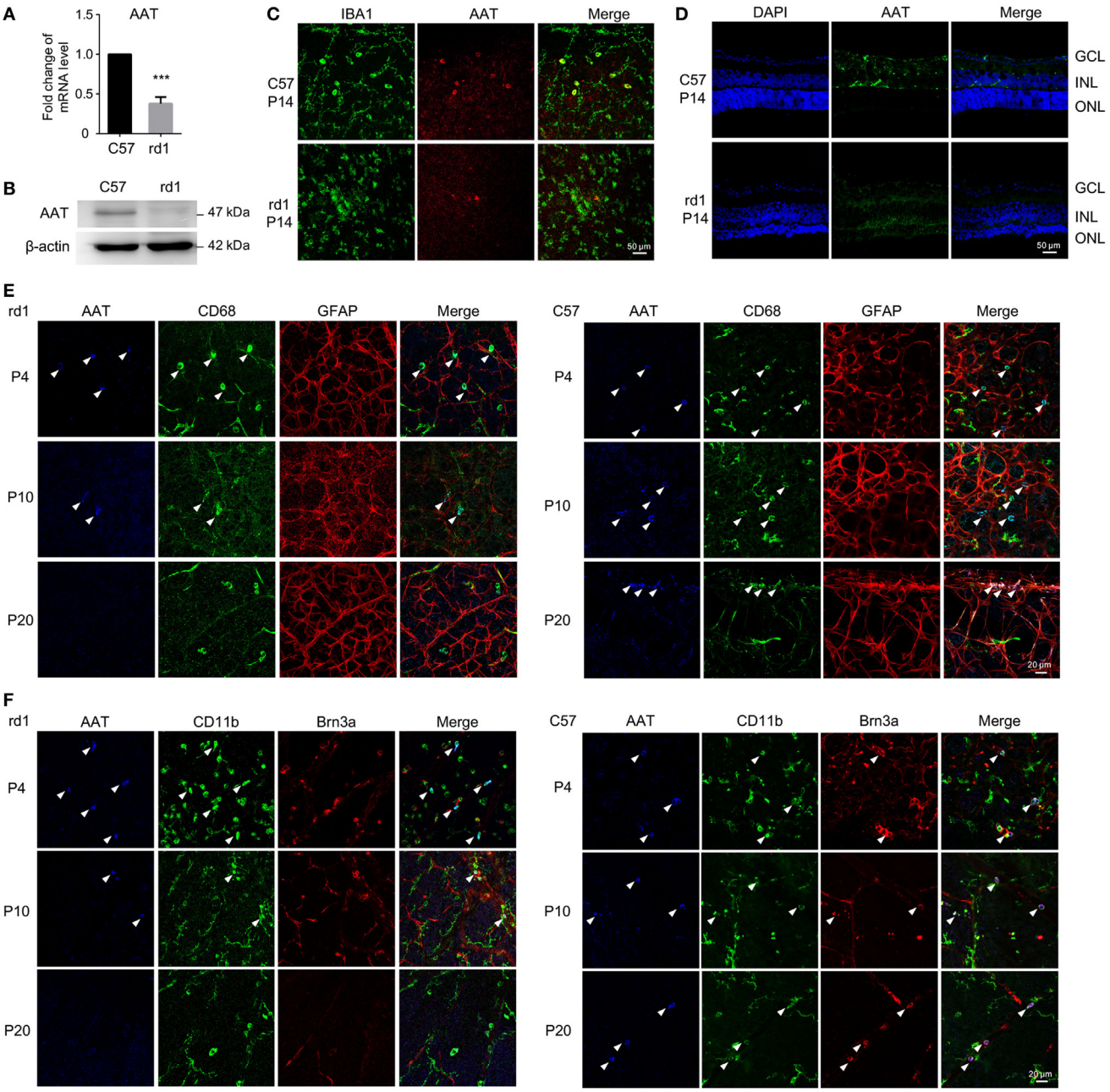

FIGURE 1 | Alpha-1 antitrypsin (AAT) expression decreased in rd1 mice. **(A,B)** Real time PCR **(A)** and western blot **(B)** results revealed that the expression of AAT decreased in retina from rd1 mice compared with C57 controls at P14. ***$p < 0.001$ (two-tailed unpaired *t*-test). **(C)**. There were some cells co-staining with AAT (red) and IBA1 (green) in C57-retina, whereas lack of AAT was observed in the retina from rd1 mice at P14. **(D)** Immunofluorescence on retinal section showed that AAT was mainly distributed in the inner nuclear layer in C57 mice, while the rd1 mice lacked AAT expression. **(E,F)** In retinal whole mounts, expression of AAT was decreased as the disease progressed from P4 to P20 in rd1 mice, whereas C57 mice presented stable expression of AAT during the same time. Of note, AAT was co-labeled mostly with CD68 **(E)** and CD11b **(F)**, also markers for microglia in the retina, slightly with Brn3a$^+$ retinal ganglion cell **(F)**, but not with GFAP$^+$ astrocytes/Müller cells **(E)**. Scare bar, 50 μm.

levels of AAT were reduced significantly in rd1 retina compared with the C57 controls at P14 when photoreceptors experienced severe loss (23, 24). Furthermore, the high expression of AAT in C57-retina was localized mainly on IBA1$^+$ microglia, whereas low level was detected on rd1-microglia at P14 (**Figure 1C**). The immunostaining on retinal cryosection showed that AAT was mostly distributed along the INL (**Figure 1D**). To further depict temporal expression of AAT on other retinal cell types, we performed co-staining of AAT and markers for several retinal cell types at different time-points. Strikingly, decreased expression of

AAT was observed in rd1 retina as the disease progressed from P4 to P20 and its expression also reduced markedly compared with the same age C57 controls, whereas both mice presented similar levels of AAT at P4 (a few days before the onset of rod photoreceptor degeneration at P9 in rd1) (**Figures 1E,F**). Of note, AAT co-labeled mostly with CD68 and CD11b, markers for microglia in the retina, while slightly with Brn3a$^+$ retinal ganglion cell (RGC), but not with GFAP$^+$ astrocytes/Mullers (**Figures 1E,F**), indicating that microglia was possibly the predominant cell type expressing AAT in the retina.

AAT Supplement Attenuated the Degenerative Retina of rd1 Mice

Given the decreased level of AAT in rd1 mice, we proposed that AAT supplement might be an alternative intervention for retinal degeneration. To test the possibility of this hypothesis, we injected 80 mg/kg AAT intraperitoneally in rd1 mice every other day from P4 to P14, and evaluated the retinal structure and thickness using SD-OCT and histologic analysis at P16. Twenty-five linear scans within 6 × 3 mm^2 area adjacent to optic nerve disk were obtained at nasal and temporal retinae (**Figure 2A**). As depicted in the 3D imaging of **Figure 2B**, the average retinal thickness was measured within a circle area, with 1.5 mm in radius, centering at 3 mm away from optic nerve disk. As expected, C57 mice presented normal retinal structures with multiple layers, including the nerve fiber layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, ONL, external limiting membrane (ELM), inner segment/outer segment (IS/OS), and RPE. However, these retinal layers were hard to distinguish the PBS-treated rd1 mice with poorly visible ONL, ELM, and IS/OS junctions. Of note, the rd1 mice exhibited a low-reflecting ONL layer after AAT supplement (**Figure 2C**). The retinal volume and thickness were calculated and mild increment could be observed in rd1 mice after AAT supplement (**Figures 2D,E**). Histologic analysis was also performed and representative sections were shown in **Figure 3A**. A thicker retina was observed in AAT-treated retinae at P16. Despite the retinal thickness was slightly elevated in the rd1 mice treated with AAT compared to those under PBS treatment, the increased amount of photoreceptor nuclei in the ONL layer was more significant (**Figures 3B,C**), indicating the pronounced loss of photoreceptors in rd1 mice was ameliorated by AAT.

AAT Supplement Improved Retinal Function From Decrement in rd1 Mice at P16

Next, the retinal function was accessed by ERG at P16. The C57 control mice displayed typical a- and b-wave responses, whereas the PBS-treated rd1 mice exhibited virtually flat signal recordings under a variety of scotopic testing conditions. After AAT supplement, recognizable a- and b-waves were recovered, though the ERG responses were still diminished in comparison to C57 mice (**Figure 4**). Average amplitudes of a- and b-wave for each group of rd1 mice were analyzed. Both a- and b-wave amplitudes were found to be protected with increased amplitudes in the AAT-treated ones, confirming the extinct ERG responses in rd1 mice could be recovered after AAT supplement.

Inherited Photoreceptor Degeneration Was Protected by AAT

To further explore whether the AAT preserved the degenerative photoreceptors, rhodopsin and TUNEL staining was performed on retinal sections. More rhodopsin-positive rod photoreceptors were identified in the ONL of AAT-treated rd1 mice compared with the PBS control (**Figure 5A**). TUNEL-positive cells were

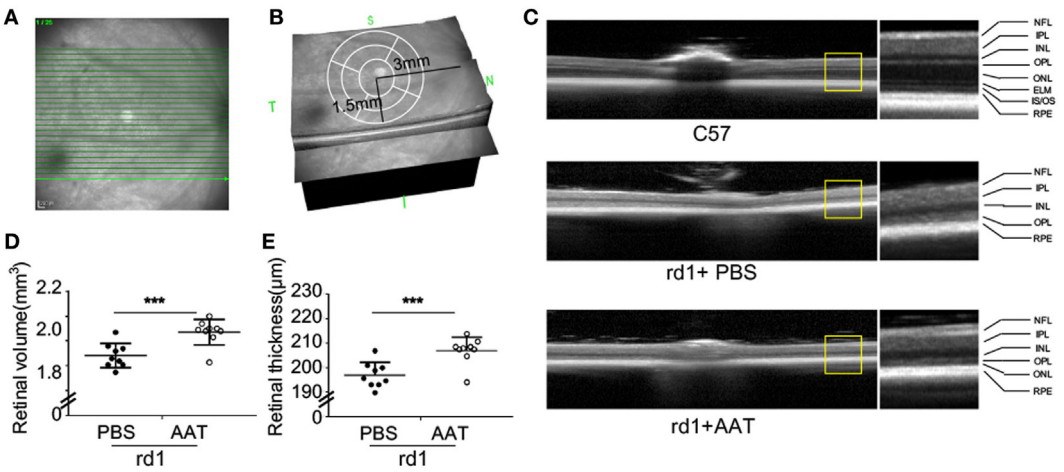

FIGURE 2 | Alpha-1 antitrypsin (AAT) reduced retinal degeneration in rd1 mice. **(A)** The scanning model used in the study. Twenty-five linear scans within 6 × 3 mm^2 area adjacent to optic nerve disk were obtained at nasal and temporal retinae. **(B)** The average retinal thickness was measured in circle area with a radius of 1.5 mm, centering at 3 mm away from optic nerve disk. **(C)** The retinal structure was well-organized with multiple layers in the C57 mice (n = 6), while the layers of ONL, ELM, and IS/OS junction were poorly visible in the PBS-treated rd1 mice at P16. However, the AAT-treated rd1 displayed a visible low-reflecting ONL layer. Abbreviations: NFL, nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; ELM, external limiting membrane; IS/OS, inner segment/outer segment; RPE, retinal pigment epithelium. The retinal volume **(D)** and thickness **(E)** were increased in the AAT-treated rd1 mice compared to PBS-treated ones. n = 9 mice for each group. ***p < 0.001 (two-tailed unpaired t-test).

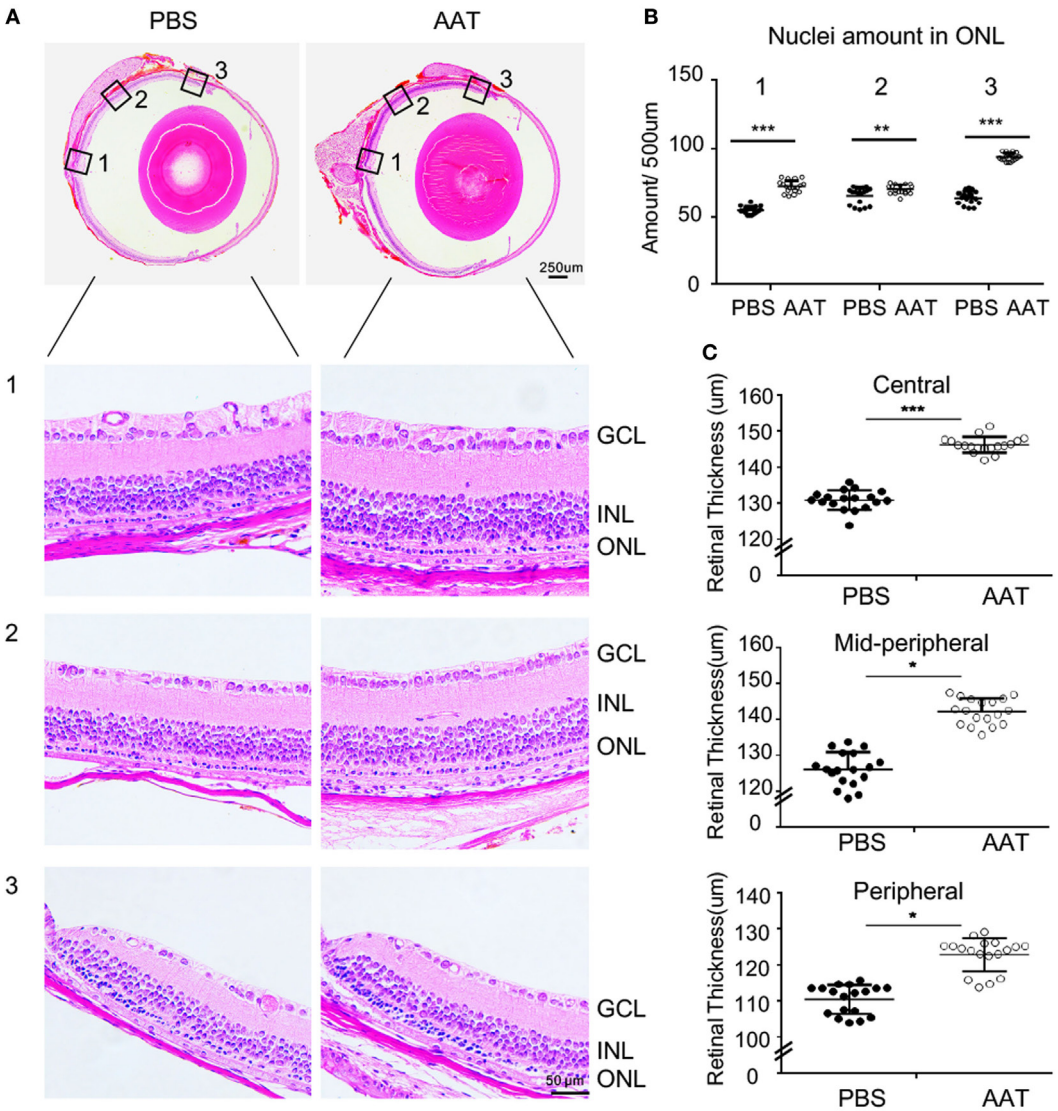

FIGURE 3 | Alpha-1 antitrypsin (AAT) alleviated the decrement of retinal thickness in rd1 mice. **(A)** Panoramic view of the central [Location 1], mid-peripheral [Location 2], and peripheral [Location 3] areas in the retinal paraffin sections by H&E staining. The representative images showed denser nuclei in the outer nuclear layer (ONL) and thicker retinal thickness of the rd1 mice treated with AAT compared to those with PBS treatment at P16. Scare bar, 50 μm. **(B)** The amounts of cellular nuclei in ONL at indicated locations were elevated significantly after AAT treatment in comparison with PBS controls. **(C)** The total thicknesses of retina were increased in the center, mid-periphery, and periphery areas in AAT-treated rd1 mice, compared with those in PBS-treated controls. Six sections of each retina and three retina of each group were used for analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed unpaired *t*-test).

evident in the ONL of PBS-treated rd1 mice at P16, while scarce TUNEL-positive cells were observed in the AAT-treated ones (**Figures 5A,B**), indicating the protective effect of AAT on the apoptotic rods.

AAT Suppressed Pro-Inflammatory M1 Microglial Polarization During Retinal Degeneration

Microglia was recognized as the main immune cell in retina and its activation contributed to neuroinflammation during retinal degeneration (10, 25). In this study, we noticed decreased number of IBA1^{+} positive microglia in the AAT-treated rd1 retina, especially in the degenerative photoreceptor layer of ONL (**Figures 5A,C**). Since the activated microglia displayed different phenotypes at various conditions, we detected the pro-inflammatory M1 phenotype at the setting of degenerative retina in rd1 mice. CD16/32 is widely used as a classic marker for M1 microglia. As demonstrated in **Figure 6A**, AAT supplement obviously decreased the ratio of CD16/32^{+}IBA1^{+} M1 microglia compared with that of the PBS-treated ones, prominently at the peripheral retina. In retinal sections, CD16/32^{+}IBA1^{+} microglia

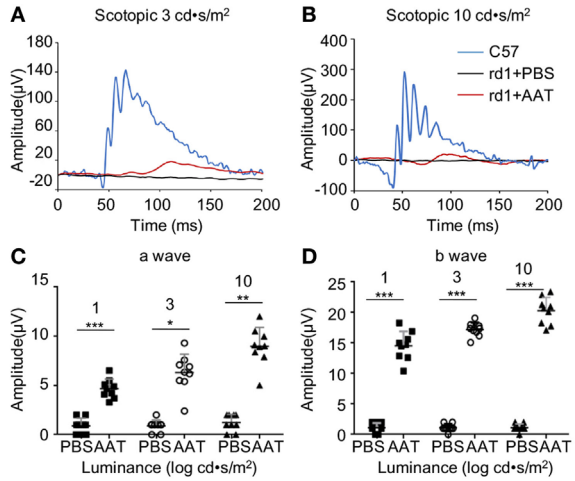

FIGURE 4 | Alpha-1 antitrypsin (AAT) supplement protected retinal function in rd1 mice. **(A,B)** Retinal function was measured at P16 by electroretinogram using single-flash recordings at light intensities of 3.0 and 10.0 log cd•s/m², respectively. C57 mice presented with typical a- and b-wave responses ($n = 6$), while the PBS-treated rd1 mice showed nearly undetectable amplitude of a- or b-waves under a variety of scotopic testing conditions. **(C,D)** AAT treatment induced mild increase in a-wave but significant elevation of b-wave amplitudes in the rd1 mice. $n = 9$ mice for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed unpaired t-test).

accumulated in the ONL of PBS-treated mice, whereas a lack of this pro-inflammatory microglia was observed in the AAT group (**Figure 6B**). In cultured BV2 microglia under oxidative stress by hydrogen peroxide stimulation, which mimic the general injury in the rd1 retina, AAT supplement also downregulated the expression of pro-inflammatory marker CD16/32^{+} on microglia (**Figure 6C**). These results suggested that AAT suppressed the pro-inflammatory M1 microglia during retinal degeneration.

Microglia Skewed Toward Anti-Inflammatory M2 Phenotype in the Presence of AAT Supplement

The effect of AAT on the anti-inflammatory M2 microglia was also evaluated. CD206 and Arg1, two classic markers for M2 phenotype, were detected by immunofluorescence on retinal whole mounts and sections. We found significantly elevated ratio of CD206^{+}IBA1^{+} microglia in retinal whole mounts from the AAT-treated group compared with the PBS-treated mice (**Figure 7A**). Similarly, although Arg1^{+}IBA1^{+} M2 microglia were absent in the PBS-treated rd1 retinae, these M2 microglia appeared after AAT supplement (**Figure 7B**). From retinal sections, we found increased CD206^{+} and Arg1^{+} microglia locating mainly in the ganglion cell layer and ONL layers (Figures S1A,B in Supplementary Material). *In vitro*, immunostaining (**Figure 7C**) and western blot (**Figure 7D**) data revealed that AAT downregulated the expression of iNOS, another typical M1 marker, and upregulated Arg1 expression. CD206 also increased in the AAT-treated BV2 microglia (Figure S1C in Supplementary Material). These data suggested that AAT could induce microglia to adopt M2 phenotype.

AAT Modulated IRF4/8 Activation and Phosphorylation of STAT1 in Microglia *In Vivo* and *In Vitro*

The interferon-regulatory factor (IRF) members are transcriptional regulators of macrophage polarization, with IRF8 and IRF4 associated with the polarization toward the M1 state and M2 state, respectively (26, 27). We next detected the expressions of IRF4, IRF8, and relevant STAT1 signaling using western blot. The data revealed that in the AAT-treated retinae from rd1 mice, IRF8 expression was significantly suppressed while the level of IRF4 was slightly increased (**Figures 8A,B**). In addition, STAT1 signaling, of which phosphorylation could induce IRF8 expression, was also inactivated with the lack of p-STAT1 after AAT supplement (**Figures 8A,B**). Similarly, AAT stimulation downregulated the expression of IRF8 and upregulated IRF4 level in the cultured primary microglia. In addition, the phosphorylation of STAT1 was inhibited in the AAT-treated primary microglia cells (**Figures 8C,D**). Thus it is possibly that AAT exerted its modulatory effect through regulating the bias of IRF4 and IRF8 expression and STAT1 activation.

DISCUSSION

Neuroinflammation plays a key role in the process of RP (9, 10, 28). In the present study, the efficacy of AAT, an old drug showing new tricks of immunomodulatory property, was evaluated for preventing the neuroinflammation during the pathogenesis of retinal degeneration in rd1 mice. Our data provided evidence for the first time that AAT could suppress the neuroinflammation and attenuate neurodegeneration in the rd1 mice through the induction of anti-inflammatory M2 microglia, underlining that an immunomodulatory therapy may be an efficient strategy to retard retinal degeneration.

Alpha-1 antitrypsin, a 52-kDa glycoprotein encoded by the Serpina gene, is produced mainly by hepatocytes and maintained in circulation (22). Actually, the circulating AAT could be transported by vascular endothelial cells in an endocytosis pathway (29, 30). It is likely that AAT supplement by intraperitoneal injection enter retina through the blood–retinal barrier. Interestingly, AAT could be produced locally by mononuclear phagocytes in tissues and the expression is often reduced in a context of inflammation (21). For example, the amount of AAT in NOD diabetic islet was significantly less than that in C57BL/6 mice (12, 16, 17). In this study, we also found reduced expression of AAT in the rd1 retina, which was associated with neuroinflammation. So it is reasonable and promising of AAT supplement to treat RP due to the decreased level of AAT.

Accumulating evidence indicated that AAT exerted a novel immunomodulatory effect at the setting of inflammation. It is currently available and is used as medication in clinical trial for treating individuals with recent onset of type 1 diabetes and graft-versus-host disease, based on its potent immunomodulatory property (31, 32). Besides, AAT exhibits notable safety profile in clinical usage (33). Hence, it merits further investigation in other settings and our data of its efficacy in RP provide evidence to extend its usages in attenuating inflammation and protect the

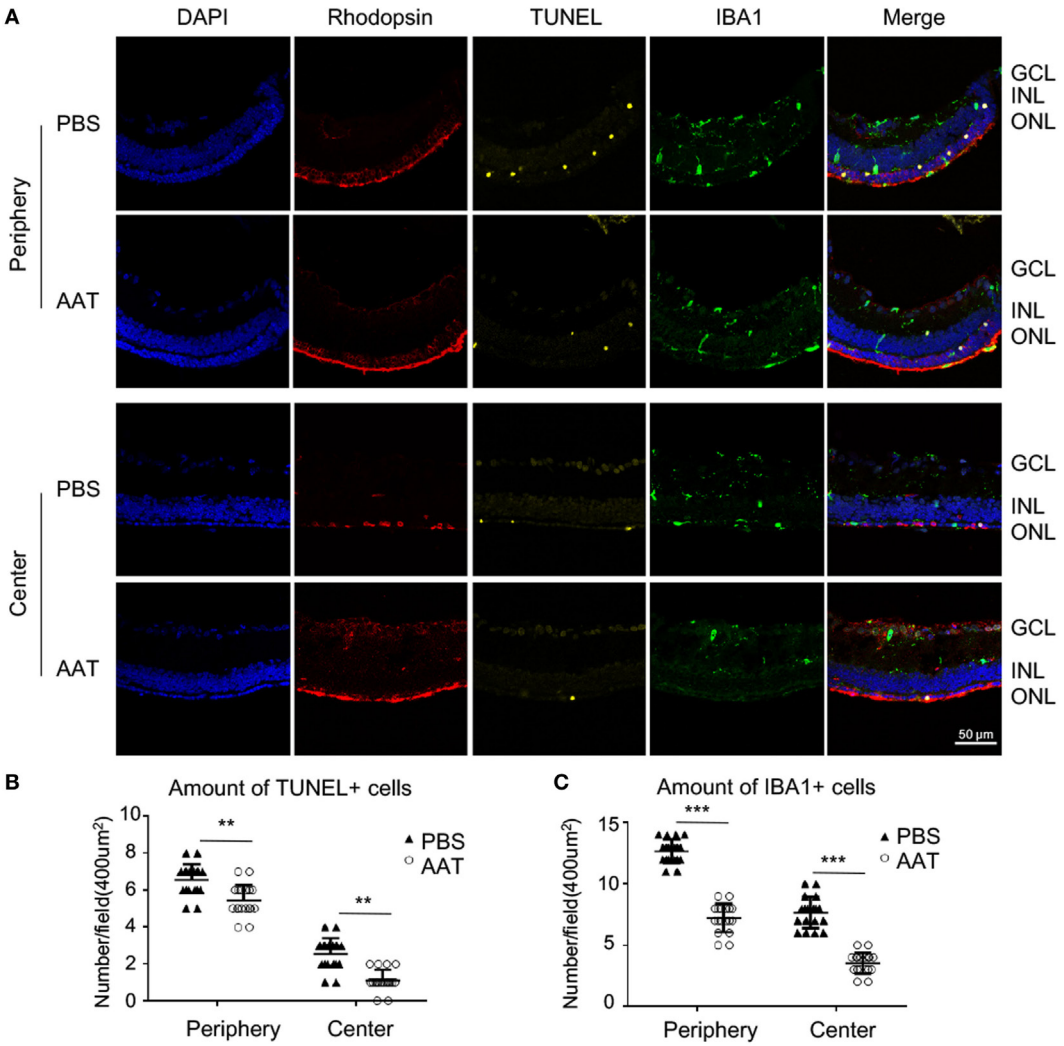

FIGURE 5 | Protective effect of alpha-1 antitrypsin (AAT) on degenerative rods. **(A)** The representative images of immunostaining on retinal sections revealed that rhodopsin-positive rod photoreceptors were scattered in the PBS-treated rd1 mice, but widely distributed and well-organized in the outer nuclear layer after AAT treatment. The TUNEL$^+$ apoptotic cells decreased in AAT supplement group, whereas the amount of IBA1$^+$ microglia also decreased comparing with the PBS-treated ones. The location "Center" refers to the panoramic view area 1 in **Figure 3A**, and "periphery" refers to the area 3. Scare bar, 50 μm. Statistical analysis of the TUNEL$^+$ **(B)** and IBA1$^+$ cells **(C)** revealed that all their amounts significantly decreased either in the center or periphery. Six sections of each retina and three retina of each group were used for analysis. $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed unpaired *t*-test).

degenerative retina. In addition, it is well known that rd1 mice, an acute autosomal recessive form for human RP, carries a mutation affecting the expression of β subunit of phosphodiesterase 6, leading to the accumulation of cGMP that is thought to trigger photoreceptor degeneration in a short time. So these mice are hard to get complete recovery by intervention with biological agents. Despite the effect of immune therapy was not as remarkable as that of gene therapy in the rd1 mouse, suppression of neuroinflammation by AAT would provide a suitable microenvironment for cell survival and a stable treatment window for other therapies, considering the fact that neuroinflammation is associated with various type of RP, it serves as a hallmark of common pathological process of RP.

Alpha-1 antitrypsin exerted its immunomodulatory effect on many cellular targets (32, 34). Previous studies showed that neutrophils and monocyte/macrophage could produce more IL-10 and reduced the generation of TNF-α after AAT stimulation (35). AAT could also regulate the activities of NK cells through the interaction of NK cells and dendritic cells (DC) (34). It is worth noting that AAT modifies the phenotype of DC and B lymphocytes toward a tolerogenic pattern, exhibiting the immunomodulatory potential (36). In the present study, retinal microglia in rd1 mice experienced M2 polarization after AAT supplement, providing a novel immunological cellular mechanism by which AAT exerts its pluripotential anti-inflammatory effects. Despite the retina was believed to be immune privilege,

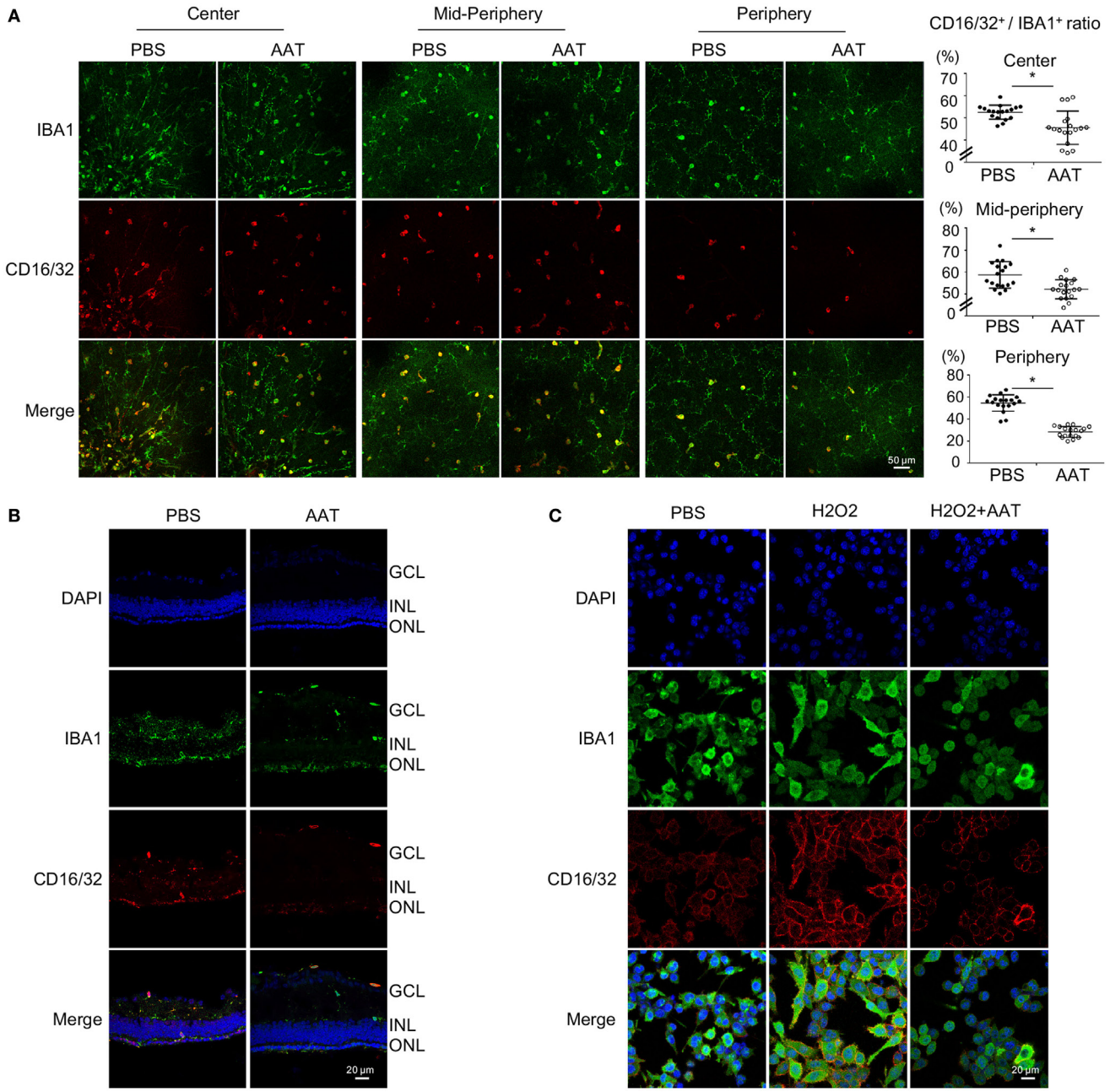

FIGURE 6 | Alpha-1 antitrypsin (AAT) suppressed pro-inflammatory M1 microglial polarization during retinal degeneration. **(A)**. In retinal whole mounts, the amount of CD16/32$^+$IBA1$^+$ M1 microglia in the central, mid-peripheral, and peripheral retina decreased significantly after AAT supplement. Scare bar, 50 μm. Depicted is mean ± SEM of three fields/eyes from six eyes. *$p < 0.05$ (two-tailed unpaired *t*-test). **(B)** In the retinal section, CD16/32$^+$IBA1$^+$ microglia prominently accumulated in the outer nuclear layer in PBS treatment group whereas the amount of CD16/32$^+$IBA1$^+$ pro-inflammatory microglia significantly decreased in the AAT-treated group. Scare bar, 20 μm. **(C)** In the cultured microglia under oxidative stress by hydrogen peroxide stimulation, AAT supplement significantly suppressed the pro-inflammatory M1 phenotype of microglia, presenting with less CD16/32$^+$ cells co-stained with IBA1$^+$ cells. Scare bar, 20 μm.

its function was monitored by the active microglia, the main resident innate immune cells in CNS, who could initiate a cascade of cellular responses and orchestrate the immune condition in retinal diseases (10, 37). Similar to other immune cells like macrophages and DC, microglia adopt various phenotypes to adapt themselves to different insults (38). Our previous study has demonstrated that retinal microglia were activated and particularly polarized to a pro-inflammatory M1 phenotype at the rapid rods degenerative phase of rd1 mice (23), suggesting that M1 microglia contribute to retinal inflammation and the conversion to M2 anti-inflammatory statue seemed to be an efficient therapy for preventing retinal degeneration. Our

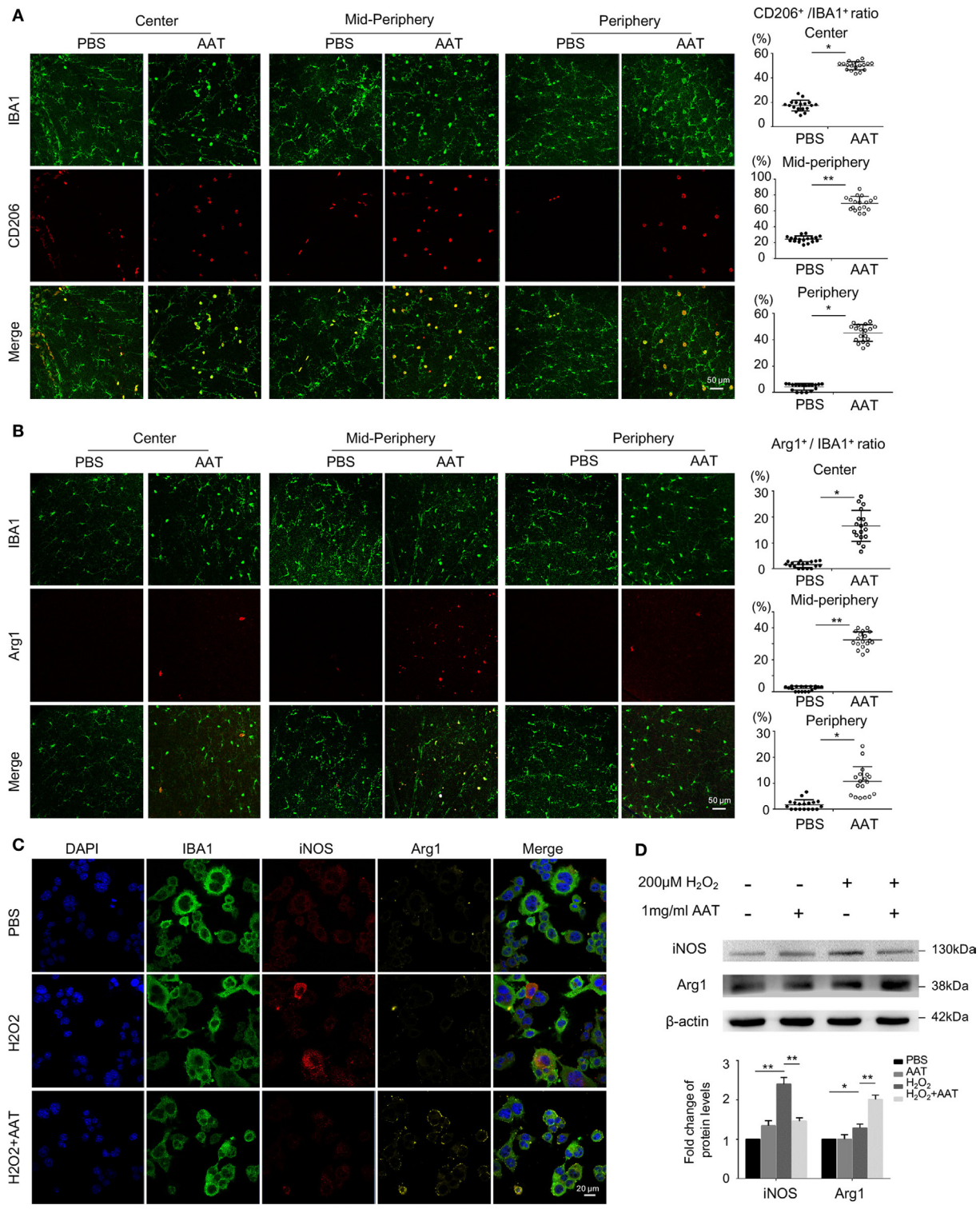

FIGURE 7 | Microglia skewed toward anti-inflammatory M2 phenotype in the presence of alpha-1 antitrypsin (AAT) supplement. **(A)** In the retinal whole mounts, the amount of $CD206^{+}IBA1^{+}$ microglia significantly increased in AAT-treated group compared with the PBS-treated mice, particularly in the central and mid-peripheral retina. **(B)** $Arg1^{+}$ cells, another M2 microglia marker, were absent in the PBS-treated rd1 retina, while after AAT supplement, these M2 microglia appeared and most concentrated in the mid-peripheral areas. Scare bar, 50 μm. Depicted is mean ± SEM of three fields/eyes from six eyes. $^{*}p < 0.05$, $^{**}p < 0.01$ (two-tailed unpaired *t*-test). **(C)** Immunostaining results showed that iNOS was elevated after hydrogen peroxide stimulation on cultured BV2 microglia, in particular expressed in the cytoplasm. In the AAT-treated microglia, not only the expression of iNOS significantly decreased, but also apparent upregulation of Arg1 expression was observed. Scare bar, 20 μm. **(D)** Western blot revealed that AAT downregulated the expression of iNOS while upregulated the Arg1 expression, the counteracting factor of iNOS.

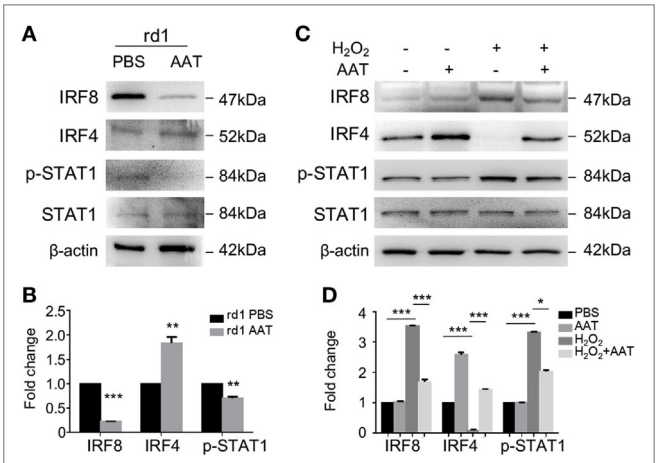

FIGURE 8 | Alpha-1 antitrypsin (AAT) modulated IRF4/8 activation and phosphorylation of STAT1 *in vivo* and *in vitro*. **(A,B)** Western blot revealed that in AAT-treated retina of rd1 mice, the IRF8 expression significantly decreased while the expression of IRF4 increased slightly. Moreover, the STAT1 signaling was suppressed after AAT supplement. **(C,D)** *In vitro*, hydrogen peroxide stimulation upregulated the expression level of IRF8 and promoted phosphorylation of STAT1 in the primary cultured microglia, while AAT treatment reversed the expression trend with decreased IRF8 and STAT1 and increased IRF4. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed unpaired *t*-test).

findings suggested that AAT upregulated M2 anti-inflammatory activity and ameliorated the retinal degeneration, offering a novel immunological mechanism of AAT, particularly in the neurodegenerative diseases, in which microglia polarization is of importance. Of course, the effects of AAT on other retinal cell types could not be excluded in the rd1 mice, and it would be interesting to explore in future study.

A number of studies have implicated that transcription factors cooperatively regulated the downstream M1/M2-associated genes of macrophage/microglia (39). Among them, the IRF family could regulate maturation and activation of immune cells (40). IRF4 and IRF8 are two homologs in the IRF family whose expressions are largely restricted to lymphoid and myeloid cells. In retina, IRF4 and IRF8 were reported to be expressed mainly in microglia (26), which shared features with myeloid/hematopoietic lineage cells. Therefore, their expression and alteration in the whole retina were believed, to some extent, to be associated with microglial activities. IRF8 could induce the expressions of a large panel of M1-related genes, such as IFN-β, IL-12, iNOS, and so on (40, 41), whereas IRF4 functions as a negative regulator of TLRs signaling, associated with the polarization to M2 state (41, 42). In this study, high IRF8 and low IRF4 were observed in rd1 retina. *In vitro*, we found hydrogen peroxide insults induced a marked elevation of IRF8 and reduction of IRF4 in primary microglia, associating with more iNOS^{+} M1 and less ARG1^{+} M2 microglia, further indicating the critical role of IRF8 on M1 polarization of microglia. Strikingly, AAT altered the expression profile with lower IRF8 and higher IRF4 and induced microglia polarization toward M2 and away from M1 *in vivo* and *in vitro*, supporting the concept that AAT modulated microglia polarization partly through regulating the balance of IRF4 and IRF8 expression. A further understanding of mechanisms involved in microglia polarization and its regulation by AAT would facilitate the intervention of neuroinflammation, as well as improve and broaden the usage of AAT in neurodegenerative diseases.

In summary, we demonstrated in the present study that treatment with AAT attenuated neuroinflammation through shifting M1–M2 microglia polarization and alleviated retinal degeneration in rd1 mice. Furthermore, AAT regulated the expression level of IRF8 and IRF4, which were associated with reduced STAT1 phosphorylation, indicating that these signaling pathways were required in AAT-induced M2 polarization. Our data provide evidences of the anti-inflammatory and immunomodulatory properties of AAT and support the possibility that immunomodulatory therapy through regulating microglia M1/M2 polarization may be benefit for treating retinal degeneration.

ETHICS STATEMENT

This study was approved by the animal experimental ethics committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (authorized number: 2014-039). All the experiments were carried out in accordance with the approved guidelines of Animal Care and Use Committee of Zhongshan Ophthalmic Center and the Association Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

AUTHOR CONTRIBUTIONS

CH and XL designed and analyzed the experiments and wrote the manuscript. TZ, ZH, XZ, XS, YL, ML, and BC performed the experiments and analyzed the data. YL assisted in the experimental design and provided key research tools.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01202/full#supplementary-material.

FIGURE S1 | Alpha-1 antitrypsin (AAT) promoted the expression of M2 microglia markers. **(A,B)** In retinal section, CD206^{+}IBA1^{+} microglia could hardly be found in PBS-treated group, and after AAT treatment, they prominently accumulated in the outer nuclear layer. The Arg1^{+}IBA1^{+} microglia were also increased in AAT group. Scare bar, 20 μm. **(C)**. In the cultured microglia under oxidative stress, CD206 was rarely expressed with 200 μM hydrogen peroxide stimulation, while its expression significantly increased after AAT treatment, with more co-staining of CD206^{+} cells and IBA1. Scare bar, 20 μm.

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Role of Glial Immunity in Lifespan Determination: A *Drosophila* Perspective

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Increasing body of evidence indicates that proper glial function plays an important role in neuroprotection and in organismal physiology throughout lifespan. Work done in the model organism *Drosophila melanogaster* has revealed important aspects of glial cell biology in the contexts of longevity and neurodegeneration. In this mini review, we summarize recent findings from work done in the fruit fly *Drosophila* about the role of glia in maintaining a healthy status during animal's life and discuss the involvement of glial innate immune pathways in lifespan and neurodegeneration. Overactive nuclear factor kappa B (NF-κB) pathways and defective phagocytosis appear to be major contributors to lifespan shortening and neuropathology. Glial NF-κB silencing on the other hand, extends lifespan possibly through an immune–neuroendocrine axis. Given the evolutionary conservation of NF-κB innate immune signaling and of macrophage ontogeny across fruit flies, rodents, and humans, the above observations in glia could potentially support efforts for therapeutic interventions targeting to ameliorate age-related pathologies.

Keywords: *Drosophila*, innate immunity, glia, lifespan, neurodegeneration, phagocytosis

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INTRODUCTION

Organismal aging is a complex phenomenon resulting in the progressive decline of physiological functions and increased susceptibility to death (1). Both, genetic and environmental factors are believed to contribute to the aging process and lifespan (1–3). Work in several model organisms including the invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans* have identified genes and cellular pathways conserved through evolution that affect longevity such as the insulin-like pathway or the target of rapamycin (TOR) pathway (4, 5). Thus, these model organisms proved to be of valuable use for studying the molecular mechanisms that underlie aging.

Drosophila, the common fruit fly, is an excellent versatile model organism to investigate the interplay between innate immune function and brain physiology among the effects of this interaction to host lifespan. There is a high degree of evolutionary conservation of the molecular mechanisms of innate immunity between flies and mammals. For instance, the two *Drosophila* nuclear factor kappa B (NF-κB)-based pathways, namely Toll and Immune deficiency (IMD) share similarities with the mammalian Toll-like receptor pathways and tumor necrosis factor receptor 1 pathways, respectively (6–10). In the context of bacterial and fungal infections, activation of these pathways leads to the translocation of NF-κB factors (Relish for IMD and Dif and Dorsal for Toll pathway, respectively) from the cytoplasm into the nucleus of the cell allowing transcription and synthesis of potent antimicrobial peptides (AMPs) (10). Phagocytosis is another powerful mechanism to eliminate cellular debris or infection that has been conserved during evolution (11, 12). In mammals, phagocytosis

is mediated by cell surface receptors, which bind bacteria or apoptotic bodies either directly or via opsonins (13). In flies, several phagocytic recognition receptors have been identified on hemocytes (the fly macrophage-like cells), among which is the EGF-like repeat-containing protein Draper (12). Draper has also been implicated in the removal of apoptotic neurons during *Drosophila* nervous system development (14) and metamorphosis (15) as well as in phagocytosis of axonal debris after axonal injury (16–18). Flies have also significantly contributed to advances in studies of neurodegeneration such as the identification of novel neuroprotective genes and provided information about conserved processes required for maintaining the structural integrity of the central nervous system (CNS) (19). Moreover, several human neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease have been effectively modeled in *Drosophila* yielding insights into the molecular base of these disorders (20).

The chronic inflammatory status that accompanies human aging, also known as inflammaging, is considered a significant risk factor for many chronic pathologies including cancer, cardiovascular and neurodegenerative disorders (21). In the context of aging, increased levels of pro-inflammatory cytokines such as TNF-alpha and Interleukine (IL)-6 are found upregulated in brain tissue (22). With age, mammalian microglia, which are the brain immune cells exhibit primed profile characterized by increased activation and enhanced secretion of pro-inflammatory cytokines (23, 24). Decline in microglial function, migration and chemotaxis are also observed with age (24). For instance, microglia's engulfment capacity of amyloid-beta (Aβ) (25) or alpha-synuclein (α-Syn) (26) oligomers, whose accumulation is characteristic for Alzheimer's and Parkinson's disease, respectively, are compromised in aged animals. Moreover, activated microglia and neuroinflammatory profiles are observed in most neurodegenerative disorders including Huntington's (27), Alzheimer's (28, 29), and Parkinson's (30–32) diseases and are believed to underlie the onset, severity, and progression of these disorders (24). Similar to mammalian models, both chronic innate immune activation (4, 33) as well as decline in phagocytic activity of glia (18) are observed in the aging *Drosophila* brain. It is thus apparent that glial immunity is linked to both, healthy aging and age-dependent neurodegeneration. In the mammalian brain, under normal physiological conditions, microglia provide the first line of defense against brain injury and infection. These cells are able to sense pathogens *via* pathogen recognition receptors, activate innate immune signaling pathways, phagocytose microorganisms, and clear cellular debris (34). Microglia also have the capacity to secrete neurotrophic factors and anti-inflammatory molecules, therefore, playing a protective role in these contexts. On the other hand, the neurodegenerative process itself can trigger inflammation (34–36), leading to detrimental effects on the brain. It is, therefore, important to understand the mechanisms by which, changes in the same signaling pathway (e.g., NF-kB) lead to two distinct phenotypes, namely healthy aging associated with neuroprotection and neurodegeneration.

Glial cells are essential players in CNS development and in maintaining homeostasis in this tissue (37). Glial cells provide trophic support to neurons, regulate ionic homeostasis in the brain, and serve as immune cells that are armed to respond to injury or infection (37). Increasing body of evidence indicates that dysfunction of diverse cellular processes specifically in glial cells may have profound impact on animal's survival and, therefore, affect life expectancy. We review here recent discoveries of the role played by glial cells in animal's lifespan, as well as how glial innate immune pathways relate to organismal longevity in the model organism *Drosophila melanogaster*.

GLIAL TYPES AND THEIR CONTRIBUTION TO HEALTHY AGING

Glial cells play major roles in nervous system development, synapse formation, plasticity, and brain homeostasis (38, 39). Five major morphologically distinct classes of glial cells with diverse functions can be appreciated in the brain of adult *Drosophila* (38–40) and additional glial subtypes in its visual system (40, 41). Among brain glia, perineurial and subperineurial glia form the blood–brain barrier (BBB) to isolate the brain from the potassium-rich hemolymph (insect blood) assuring optimal neuronal function (42–44). To meet the high-energy demands of neurons, glia supply neurons with metabolites through an evolutionary conserved process known as metabolic coupling (45). *Drosophila* BBB glia support neurons by providing them metabolic factors derived from the break down of the sugar trehalose (45). Glia-, but not neuron-specific silencing of the genes encoding the glycolytic enzymes *Trehalase* and *Pyruvate kinase* substantially shortens flies' lifespan and leads to neurodegeneration (45). Interestingly, glia-specific knock down of another gene encoding an enzyme involved in glycolysis, *Aldolase*, leads to neurodegeneration and shortened lifespan (46). Together, these studies indicate that glial glycolysis plays an important role in healthy aging and neuroprotection. Along the same lines, mutations in the gene encoding the glia-enriched monocarboxylate transporter *Chaski*, lead to shortened lifespan, synaptic dysfunction, and locomotor impairment pointing to an important role for glial transport of metabolites during the animal's lifespan (47). This work is of particular importance as it is becoming increasingly evident that metabolic changes, among which lower brain glucose metabolism, accompanies aging and Alzheimer's disease in humans (48). Metabolomics analysis of mouse brain samples reveals compromised energy state in the aging brain, possibly affecting glial cells that supply glycolytic substrates to neurons (49).

Ensheathing glia, which express the engulfment receptor Draper are the main subtype of adult *Drosophila* glial cells that phagocytose axonal debris following nerve injury (50). Cortex glia play an important trophic role for neurons in the adult brain (38) and are important for neuronal excitability as downregulation of a potassium-dependent sodium/calcium exchanger in this glial subtype leads to seizures in the adult (51). Astrocytes, which share morphological and functional properties with mammalian astrocytes (38) are major contributors to the maintenance of neurotransmitter homeostasis and are involved in regulating circadian rhythmicity in the adult (52). In the *Drosophila*, adult visual system several glial subtypes among, which epithelial glia play a role in synaptic transmission and the processing of

visual information (41). It was recently reported that lipid droplet accumulation in these glia due to mitochondrial dysfunction in neurons contributes to neurodegeneration (53). The recently discovered Semper glia, which share functional features with mammalian visual system glial cells such as Müller glia, astrocytes, and oligodendrocytes provide support to photoreceptors and prevent light-induced retinal degeneration (40).

Another, unique, microglia-like glial subtype has been recently discovered in *Drosophila*. These cells, called MANF immunoreactive cells (MiCs) are transiently present in the metamorphosing pupal brain, but not in the adult stage, and upon certain conditions in glia, including *(i)* genetic silencing of dmMANF (Mesencephalic astrocyte-derived neurotrophic factor), *(ii)* induction of autophagy via overexpression of *Atg-1* or the dominant-negative form of Target of rapamycin (TOR^{TED}), and *(iii)* challenge of innate immunity by ectopic expression of IMD-pathway receptors *PGRP-LC* or *PGRP-LE* (54). Although MiCs have features reminiscent of mammalian microglia, they were not observed in brains of 10-day-old fly mutants that exhibit neurodegeneration such as ATM^{8} (discussed later in the text) and *swiss cheese* or of *Drosophila* α-Syn model of Parkinson's disease. It appears that MiCs are immunoreactive; they express the NF-κB transcription factor Relish in their nucleus as well as the phagocytic receptor Draper on their surface (54). Interestingly, glia-specific silencing of *dmMANF* also leads to neurodegeneration and shorter lifespan in the adult suggesting a homeostatic role for this gene in glia (55). How exactly MiCs contribute to this phenotype is not known. More studies are needed in order to fully characterize this intriguing glial cell population and how exactly they relate to brain immune function and healthy lifespan. Glial subtypes and their function in the adult are presented in **Table 1**.

TABLE 1 | Glial subtypes and their location and functions in the adult.

Glial subtype	Function in adult	Location	Reference
Cortex glia	– Trophic support to neurons – Regulation of seizure susceptibility	– Brain cortex – Wrap neuronal cell bodies and processes	Kremer et al. (39) Stork et al. (56) Melom et al. (51)
Astrocyte-like glia	– Maintenance of neurotransmitter homeostasis – Circadian rhythm regulation	– Brain neuropil	Kremer et al. (39) Rival et al. (57) Stork et al. (58) Suh et al. (59) Ng et al. (52)
Ensheathing glia	– Phagocytosis of debris after injury – Regulation of olfactory circuit plasticity	– Brain neuropil – Associated with axon tracts	Kremer et al. (39) Doherty et al. (50) Kazama et al. (60)
Perineurial glia	– Blood–brain barrier (BBB) formation and chemoisolaion – Sugar import into the CNS	– Brain surface	Kremer et al. (39) Featherstone (44) Hindle et al. (43) Miller et al. (46) Volkenhoff et al. (45)
Subperineurial glia	– BBB formation and chemoisolaion	– Brain surface	Kremer et al. (39) Featherstone (44) Hindle et al. (43)
MANF immunoreactive cells	– Microglia-like cells	– Pupal brain neuropil	Stratoulias et al. (54)
Adult visual system glia	– Role in synaptic transmission – Prevent light-induced retinal degeneration	– Optic lobe	Chotard et al. (41) Charlton-Perkins et al. (40)

ROLE OF GLIAL IMMUNITY IN NEURODEGENERATION AND SHORTENED LIFESPAN

Prolonged activation of inflammatory responses often translates into harmful consequences ultimately leading to reduction of animal's lifespan. In the context of brain aging, persistent IMD pathway activation, as well as defective glial clearance function are associated with neurodegenerative phenotypes. Several studies in *Drosophila* highlight the role of glial innate immune responses (phagocytosis as well as NF-κB activation) in promoting neurodegeneration and shortening lifespan. One of the first reports correlating glial activation of the NF-κB ortholog Relish with neurodegenerative phenotypes and lifespan shortening is a study done in a fly model of Ataxia-telangiectasia (A-T) (61). This work shows that glial cells in the fly brain are responsible for increased innate immune activation when ATM kinase activity is reduced. AMPs, which are direct NF-κB transcriptional targets, are up regulated exclusively in glial cells in ATM^{8} mutants leading to Caspase-3 activation in neighboring neurons suggesting that neurodegeneration could be driven by increase in glial immunity (61). Flies in which *ATM* is specifically silenced in glial cells exhibit shortened lifespan, premature defects in locomotor activity, and spongiform brain pathology in conjunction with activation of Caspase-3 indicative for neurodegeneration (61). Results from a subsequent study done by the same group show that the degree of activation of the innate immune response correlates with the severity of neurodegeneration and lifespan duration in ATM^{8} mutants and that glial overexpression of a constitutively active form of Relish (Rel-D) leads to neurodegeneration (62). The fact that innate immune activation in brain tissue contributes to neuropathology is supported by findings from other groups that implicate both, activation of the IMD and Toll pathways in *Drosophila* models of light-induced retinal degeneration and Alzheimer's disease, respectively (63, 64). Pan-neuronal activation of constitutively active Relish results in increased lethality at eclosion pointing to a toxic effect of prolonged immunity in nerve cells (63); however, glial-specific effects of innate immune activation on lifespan and neurodegeneration in these models remains to be determined.

Another piece of evidence for direct implication of the NF-κB-dependent innate immune response in neurodegeneration and longevity comes from the finding that mutation in *defense repressor 1* (*Dnr1*), a negative regulator of the IMD pathway acting at the level of the caspase Dredd, leads to progressive neurodegeneration and reduced lifespan in a Relish-dependent manner (65).

In the same study, the authors report that bacterially induced progressive neurodegeneration and resulting locomotor defects are suppressed when *Relish* is specifically knocked down in glial cells. This work goes further by demonstrating that glia-specific overexpression of several AMPs also leads to progressive neurodegeneration. Intriguingly, the overexpression of single AMP-coding genes in glia is sufficient to cause neuropathology and this effect is direct because glial knock down of *Relish* does not suppress *Defensin-* nor *Drosomycin*-induced neurodegeneration (65). A subsequent study demonstrates that glia-specific overexpression of individual AMPs results in impaired locomotor activity and shortened lifespan providing additional evidence for the effect of these NF-κB target genes on fitness and longevity (33).

The fly IMD pathway is tightly regulated at almost every step of the signaling cascade from the surface to the nucleus of the cell (8, 66). In addition to *Dnr-1*, mutants for other intracellular safeguards of the pathway such as *Pirk, Trabid,* and *Transglutaminase* (*Tg*) that act at the level of the adaptor protein Imd, the kinase TAK1, and the transcription factor Relish, respectively, also exhibit shortened lifespan and neurodegeneration along with brain-specific upregulation of AMPs (33) (**Figure 1**). Glial silencing of *Relish* in *Trabid* mutants suppresses the age-dependent locomotor impairments and neurodegeneration in these flies and also restores lifespan to almost wild-type levels (33). Altogether, these studies attribute a role for glial NF-κB activation and downstream effectors such as the AMPs in lifespan and neurodegeneration.

Equally important to the overactive NF-κB/Relish branch of the immune response, are the alterations in glial phagocytosis, which are also associated with enhanced neurodegeneration and reduction in lifespan. A recent report showed that while protein levels of the engulfment receptor Draper are reduced in an age-dependent manner, glia's efficiency in removing cellular debris, such as the ones deriving from degenerating neurons, declines (18). In the context of healthy aging, reduced Draper levels follow an age-associated regression of glial phosphoinositide-3-kinase signaling that mediates TOR-dependent translation of *draper* mRNA (**Figure 1**) while in situations of neuronal injuries a STAT92E-dependent transcriptional upregulation of *draper* has

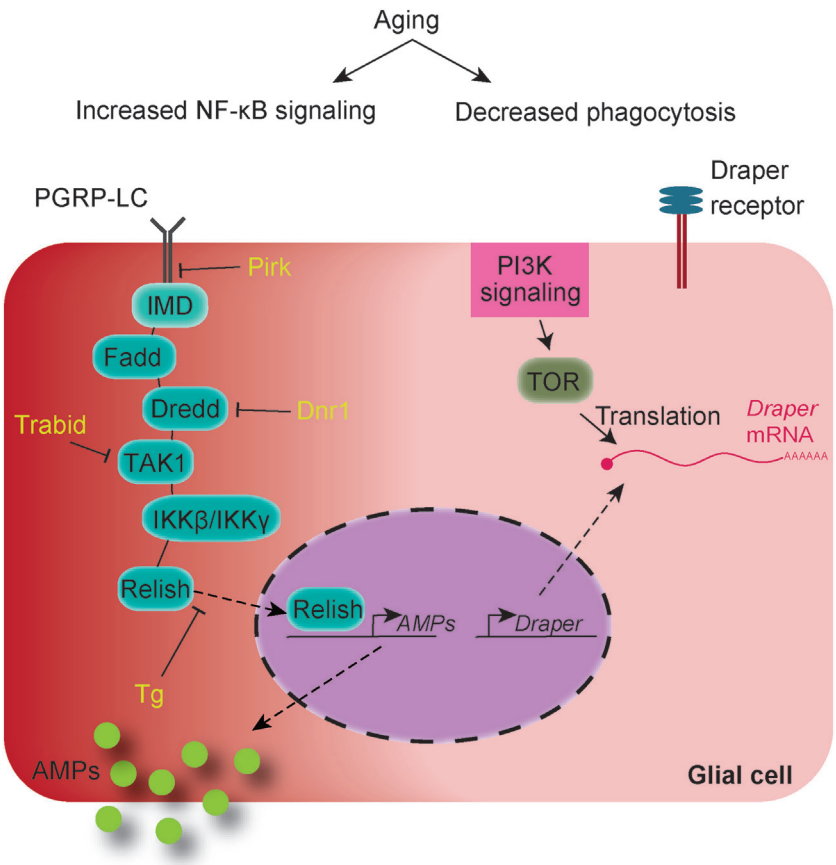

FIGURE 1 | Age-dependent changes in innate immune pathways in *Drosophila* glial cells: immune deficiency (IMD) pathway (on the left) shows age-dependent activation resulting in increased levels of antimicrobial peptides (AMPs) in middle and old-aged adults in absence of microbial challenge. Mutations in genes encoding specific IMD negative regulators namely *Dnr1*, *trabid*, *Transglutaminase (Tg,)* and *pirk* release the pathway allowing activation of Relish and subsequent transcription of downstream genes including those encoding AMPs. Aging also affects expression of the phagocytic receptor Draper (on the right) leading to inefficient phagocytic capacity of glial cells. Draper expression levels in the healthy brain are regulated *via* phosphoinositide-3-kinase signaling activity that mediates TOR-dependent translation of *draper* mRNA in glial cells. Age related decline in the activity of this signaling cascade leads to reduction in protein levels of Draper in glia.

been described (18, 67). Moreover, flies mutant for Draper exhibit short lifespan (68) and age-dependent neurodegeneration (69), pointing to a neuroprotective role for the phagocytic branch of the innate immune response. Flies, in which *Draper* is silenced specifically in glial cells, also exhibit age-dependent neurodegeneration (69). Persistent apoptotic neurons throughout the lifespan of *Draper* mutants that are not efficiently processed by glial cells due to defects in phagosome maturation appear to be the main reason for the observed phenotypes (69). However, how exactly corpse processing defects cause neurodegeneration remains to be determined.

ROLE OF GLIAL IMMUNITY IN LIFESPAN EXTENSION

Kounatidis and colleagues (33) describe an age-dependent shift in IMD-related AMP transcription in *Drosophila* including tissues like the brain of adult flies that is accompanied by neurological impairments such as decreased locomotor performance and increased neurodegeneration in 50-day-old wild-type flies (33). In the same study, the suppression of age-dependent progressive immunity by silencing three components of the IMD pathway, namely *Imd*, *Dredd,* and *Relish* in glial cells results in increased transcription of the *adipokinetic hormone* (*AKH*)-coding gene concomitant with high nutrient levels later in life and an extension of active lifespan (33). AKH is the fly ortholog of the mammalian gonadotropin-releasing hormone (70), which in mice controls an NF-κB-dependent immune-neuroendocrine axis that is involved in organismal aging (71). A remarkable deceleration of the aging process is recorded in mice upon hypothalamic blocking of NF-κB and the upstream kinase IKK-β (inhibitor of nuclear factor kappa-B kinase subunit beta), followed by an increased median longevity by nearly 20% (71). Similarly, in flies, glial-specific NF-κB immune signaling suppression results in 61% increase in median longevity compared to controls, which is also accompanied by increased locomotor activity in older age (33).

Recent studies in rodents that employ antiaging drugs link lifespan extension with reduction of age-associated overproduction of the pro-inflammatory cytokine TNF-alpha by microglia in both hypothalamus and hippocampus (72). Collectively, these studies implicate glial NF-κB signaling in lifespan determination and point to a role for the immune-neuroendocrine axis in this process.

CONCLUSION/FUTURE DIRECTIONS

It is becoming increasingly evident that glial cells play an important role in neuroprotection and in organismal physiology throughout lifespan. In the recent years, studies in the model organism *Drosophila* have revealed numerous aspects of glial contribution toward both, healthy aging and the development and progression of age-related pathologies of the nervous system. Dysregulation of glial innate immune reactions such as improper NF-κB signaling or impaired Draper-based phagocytosis results in early onset neurodegeneration and lifespan shortening. Thus, both branches of the innate immune response seem to contribute in host neuroprotection and longevity. Additional work is needed to investigate whether these two pieces of the innate immune response possess synergistic properties and identify possible cellular factors that regulate both the inflammatory and phagocytic pathways in glial cells.

Injection of Alzheimer's disease-related Aβ oligomers (AβOs) into the brains of mice and macaques results in activated pro-inflammatory IKKβ/NF-κB signaling in the hypothalamus and subsequent induction of peripheral glucose intolerance (73). The majority of dementia-related diseases share an inflammation-based branch. Given the evolutionary conservation of innate immune signaling in flies, mice, and humans, strategies like challenging the inflammatory effect of NF-κB pathways could be proved an effective strategy in both "healthy aging" status and in cases of predisposition to age-related neurological diseases.

Additional avenues for future research will involve the studies of the exact mechanisms by which glial effectors downstream of NF-κB such as the AMPs induce neurotoxicity and shorten lifespan. Amyloid-β peptides, which are involved in Alzheimer's disease pathology have antimicrobial properties (74) and can protect the mouse brain from infection with pathogenic bacteria (75). AMPs can exert bactericidal effects by inserting into and disrupting bacterial membranes (76, 77). Interestingly, modeling studies have suggested that Aβ peptides can also insert into lipid bilayers and potentially form pores in cellular membranes, and thus can damage cells and lead to neurodegeneration (78). Flies can offer an excellent experimental system to address this question and provide important new insights into the mechanisms of AMP toxicity.

Interpolations that boost glial engulfment activity can delay age-dependent processes like delayed clearance of damaged neurons and cellular debris (18). Furthermore, it has been shown that enhanced glial engulfment reverses Aβ accumulation as well as associated behavioral phenotypes in a *Drosophila* AD model (68).

Therefore, glial immune signaling will potentially provide a new cohort of molecular foci for therapeutic interventions in cases of common incurable neurodegenerative disorders in the aging population.

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IK and SC wrote the manuscript and designed the figures.

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