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Microglial heterogeneity: distinct cell types or differential functional adaptation?

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Abstract

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

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

INTRODUCTION

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

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

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

Microglial heterogeneity

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

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

Heterogeneity between brain regions

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

Heterogeneity between sexes

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

Intrinsically defined heterogeneity?

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

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

Heterogenic microglial morphology

Amoeboid vs. ramified microglia

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

Dark microglia

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

Axon initial segment-associated microglia

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

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

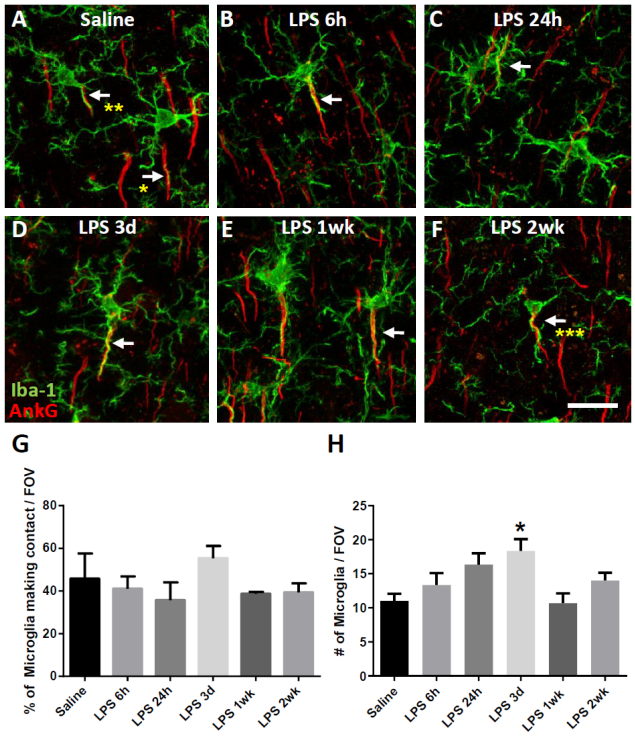


Figure 1. Frequency of microglial-AIS contact is not altered in LPS-induced neuroinflammation. Female c57black6 mice were given a single intraperitoneal injection of LPS (5 mg/kg) or vehicle (0.9% saline, 10 mL/kg). Confocal z-stacks spanning an optical thickness of 25 μm, using a pinhole of 1 Airy disc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

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

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

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

Heterogenic microglial transcriptomes

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

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

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

Disease-associated microglia

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

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

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

Heterogenic expression in inflammatory microglia

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

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

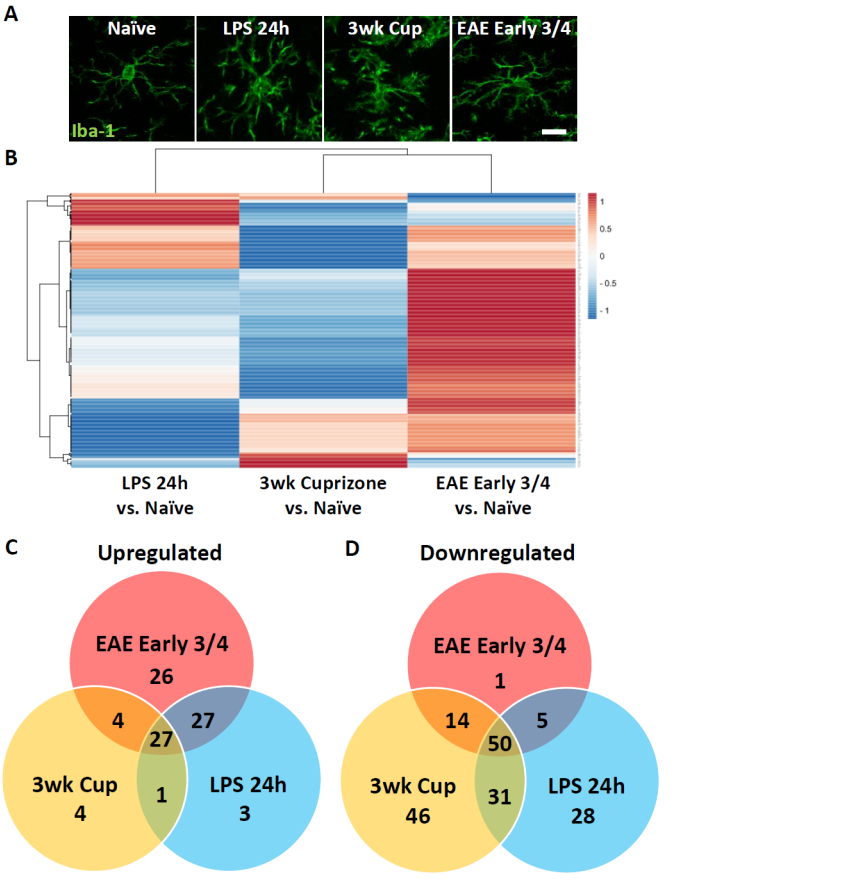
Recently, we analyzed CNS pathology in three models of neuroinflammation. In all three models, microglia presented with reactive phenotypes and these cells maintained, or even increased, contact with the AIS. However, in two of the models, the AISs were disrupted and in one, the AISs were preserved.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

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

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

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

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



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

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

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

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

Transcriptomic defined subsets of microglia

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

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

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

CONCLUSION

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

DECLARATIONS

Authors' contributions

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

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

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

Availability of data and materials

NanoString raw data files are provided in Supplementary Material.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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