Definition of a mouse microglial subset that regulates neuronal development and proinflammatory responses in the brain

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Abstract

Expression of Itgax (encoding the CD11c surface protein) and Spp1 (encoding osteopontin; OPN) has been associated with activated microglia that can develop in healthy brains and some neuroinflammatory disorders. However, whether CD11c and OPN expression is a consequence of microglial activation or represents a portion of the genetic program expressed by a stable microglial subset is unknown. Here, we show that OPN production in the brain is confined to a small CD11c+ microglial subset that differentiates from CD11c+ precursors in perinatal life after uptake of apoptotic neurons. Our analysis suggests that coexpression of OPN and CD11c marks a microglial subset that is expressed at birth and persists into late adult life, independent of environmental activation stimuli. Analysis of the contribution of OPN to the intrinsic functions of this CD11c+ microglial subset indicates that OPN is required for subset stability and the execution of phagocytic and proinflammatory responses, in part through OPN-dependent engagement of the αVβ3-integrin receptor. Definition of OPN-producing CD11c+ microglia as a functional microglial subset provides insight into microglial differentiation in health and disease.

CD11c+ microglia represents a part of a subset-specific genetic program that this CD11c+ microglial subset is a feature of microglial activation or marks a subset-specific genetic program.

Results

CD11c+ Microglia Are Formed Early in Mouse Brain Development upon Engagement of ANs Independent of Microglial Activation. In DCs, the CD11c protein represents an essential part of the iC3b heterodimeric receptor that mediates phagocytosis (1).

Significance

CD11c+ microglia enriched for osteopontin (OPN) expression appear at distinct stages of brain development, aging, and several neurodegenerative disorders. Whether coexpression of CD11c+ and OPN results from microglial activation or represents a part of a subset-specific genetic program is unknown. We find that this CD11c+ microglial subset is formed before birth upon uptake of apoptotic neurons.

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Microglia that express CD11c are detectable at birth but decrease dramatically by 3 mo of age (3). We therefore traced the development of CD11c+ microglia in healthy C57BL/6 mice from prenatal life through adulthood. We first validated microglial CD11c expression by flow cytometry. To distinguish microglia (CD11b+CD45high) and macrophages (CD11b+CD45low), we used the CCR2 marker that is up-regulated on blood-expressed macrophages but not expressed by microglia (3, 16), and a microglia-specific marker, Tmem119 (17). The CD11b+CD45high subset that expresses CCR2 but not Tmem119 was confirmed as macrophages, while CD11b+CD45low cells that all express Tmem119 but do not express CCR2 were confirmed as microglia. Fluorescence minus one (FMO) negative controls and brain CD45+ cells that do not express CD11c were included to confirm the specificity of CD11c staining in CD11b+CD45high microglial population (Fig. 1L). We note that CD11c+ microglia arise late in embryogenesis (embryonic day 18.5; E18.5), increasing to about 7 to 8% of total microglia by postnatal day 5 (P5) before reeding to almost undetectable levels (<1%) in young adult life. However, CD11c+ microglia reemerge in older (6- to 9-mo-old) mice to represent about 10% of total microglia (Fig. 1B).

Since uptake of ANs may trigger changes in the microglial genetic program that include up-regulation of inflammatory disorders, including lipopolysaccharide (LPS) and the treatment with diverse activating stimuli that mimic CNS inflammation. We observed that 23% of total CD11c staining in CD11b+CD45high microglia (Fig. 1J). We note that CD11c+ microglia arise late in embryogenesis (embryonic day 18.5; E18.5), increasing to about 7 to 8% of total microglia by postnatal day 5 (P5) before reeding to almost undetectable levels (<1%) in young adult life. However, CD11c+ microglia reemerge in older (6- to 9-mo-old) mice to represent about 10% of total microglia (Fig. 1B).

Up-regulation of CD11c expression was not simply a consequence of AN engulfment early in development. We enriched CD11c+ microglia by negative isolation with anti-CD11c magnetic beads to yield CD11c+ microglia (purity >99%) and incubated them with fluorescently labeled ANs for 72 h followed by a determination of the percentage of CD11c+ microglia that expressed CD11c. We observed that 25% of total CD11c+ microglia had ingested ANs (AN+), of which 77.4% of these AN+ microglia were CD11c+. In contrast, of the 77% of CD11c+ microglia that had not ingested ANs, <1% expressed CD11c (Fig. 1, C, Upper). These findings also indicate that ~18% of total CD11c+ microglia go on to express CD11c+ after incubation with ANs, while <1.5% of the microglia incubated for 72 h in the absence of ANs become CD11c+ (Fig. 1, C, Lower).

We then examined the interactions that might promote the formation of CD11c+ microglia in neonatal life. Phagocytic receptors expressed by developing microglia that may mediate uptake and clearance of apoptotic cells include TAM (Tyro3, Axl, MerTK) and the αvβ3-integrin receptors (20, 21). We analyzed the impact of specific inhibition of αvβ3 and TAM receptors on AN uptake and its potential impact on acquisition of a CD11c+ phenotype by CD11c+ microglia. The αvβ3 inhibitor cilengitide (Cil) or the LDC1267 inhibitor of pan-TAM receptors reduced AN uptake of CD11c+ microglia by ~50%. A combination of these two inhibitors further reduced CD11c+ microglial AN uptake to background levels (Fig. 1 F, Left). This combination of these two inhibitors further reduced CD11c+ microglial AN uptake to background levels (Fig. 1 F, Left). Consistent with the findings in Fig. 1C, we noted that stimulation by ANs induced CD11c expression by ~20% of initial CD11c+ microglia over 72 h, while CD11c expression was not detectable in the absence of AN stimulation during the same time period. Finally, inhibition of AN uptake by Cil or LDC1267 reduced acquisition of a CD11c+ phenotype by CD11c+ progenitors by about 50 to 75% in each case and inclusion of both inhibitors fully prevented this phenotypic transition (Fig. 1 F, Right). These findings suggest that engulfment of apoptotic cells may trigger a CD11c+→CD11c− transition following interaction of ANs with microglial receptors that include αvβ3-integrin and TAM.

The Stability of CD11c Expression by Microglia Is Regulated by OPN. We validated microglial OPN expression by flow cytometry analysis. We used several controls, including an isotype control and, importantly, OPN knockout (OPN-KO) microglia as negative control cells and microglia that selectively express the intracellular isoform of OPN (OPN-i knockin [OPN-iKI]) as a positive control. Wild-type (WT) microglia and OPN-iKI microglia show similar levels of OPN staining, while staining of microglia from either OPN-KO donors or microglia stained with an isotype control does not yield a detectable signal (SI Appendix, Fig. S1). We noted that microglial OPN production is confined to CD11c+ microglia and is not significantly expressed by CD11c− microglia (Fig. 2A). Moreover, AN-induced differentiation of CD11c+ to CD11c− microglia was also accompanied by a sharp up-regulation of OPN production (Fig. 2B).

To define the impact of OPN on the CD11c+ phenotype, we characterized CD11c+ microglia from OPN-KO and WT mice during early development and aging. OPN deficiency led to a ~50% reduction in the proportions of CD11c+ microglia in WT mice at P5 and 6 and 9 mo of age, according to flow cytometry analysis (SI Appendix, Fig. S2A). In situ analysis of CD11c+ and CD11c− microglia in brain cryosections of P5 and 9-mo-old WT and OPN-KO mice confirmed reduced proportions of CD11c+ microglia from OPN-KO mice (50 to 75% reduction). CD11c-specific staining was validated using negative controls (SI Appendix, Fig. S2B).

We further defined the contribution of OPN to the stability of CD11c+ expression by microglia isolated from P5 and 9-mo-old WT and OPN-KO mice during a 7-d in vitro culture period. The numbers of CD11c+ microglia were comparable between P5 and 9-mo-old WT and OPN-KO mice at day 0 (SI Appendix, Fig. S3A). OPN-deficient neonatal (P5) CD11c+ microglia showed a 75% reduction in CD11c expression (Fig. 2C), while OPN-deficient CD11c− microglia from 9-mo-old OPN-KO mice showed a ~50% reduction in CD11c expression (Fig. 2D). We examined the stability of the microglial CD11c+ phenotype in a more physiological setting using organotypic hippocampal slice cultures (OHSCs). This system allows replacement of endogenous microglia after deletion of resident microglia from organotypic hippocampal tissue slices. We repopulated these microglia-free hippocampal slices with CD11c+ microglia (>95% CD11c+) from WT or OPN-KO P5 and 9-mo-old donors. WT and OPN-KO mice showed identical numbers of CD11c+ microglia at day 0 in OHSCs (SI Appendix, Fig. S3B). The WT CD11c+ microglia retained their CD11c+ phenotype, while the majority of OPN-deficient CD11c+ microglia did not (Fig. 2E and F).

We then assessed the response of CD11c+ microglia to Aβ, a pathogenic microglial stimulus that is a prominent feature of age-related neurodegenerative disease. We observed that CD11c+ microglia from 9-mo-old WT mice stably expressed CD11c after in vitro Aβ1-42 stimulation and in OHSCs in contrast to OPN-deficient CD11c+ microglia, which lost 60 to 75% of CD11c expression (Fig. 2G and H). Taken together, these data suggest that stable expression of microglial CD11c may require coexpression of OPN.

Genetic Profiling of CD11c+ Microglia. We then asked whether CD11c+ microglia represent a distinct subset that expressed a characteristic genetic profile. To that end, we performed RNA-sequencing (RNA-seq) analysis of fluorescence-activated cell
**Fig. 1.** CD11c⁺ microglia are differentiated from CD11c⁻ precursors upon engulfment of ANs early in development independent of microglial activation. (A) Brain single-cell suspensions of 9-mo-old C57BL/6 (B6) WT mice were generated for validation of microglial CD11c expression by flow cytometry. We first gate on CD11b⁺ cells from single/live cells followed by gating of CD11b⁺CD45low populations as potential microglia. Almost all the cells in this population (∼99%) are Tmem119⁺ while CCR2 expression is not detectable. In contrast, CCR2 but not Tmem119 expression is detected in the CD11b⁺CD45high fraction, namely putative macrophage populations. Analysis of the CD11b⁺CD45low microglial population using FMO negative controls for these flow cytometry plots confirms specificity. Brain CD45⁻/C0 cells, mainly containing nonimmune cells (neurons, astrocytes, and oligodendrocytes but not microglia) that do not express CD11c were used as negative controls to further validate the specificity of this strategy. (B) The proportion of CD11c⁺ microglia in WT mice during early development and normal aging (n = 3) was determined by flow cytometry analysis. CD11c⁺ microglia were initially present at E18.5, peaked at P5, and gradually declined to marginal levels in young adulthood (3 mo) followed by reemergence and further expansion during aging. (C) CD11c⁻ microglia were isolated from P5 WT mice by negative selection with anti-CD11c magnetic beads (purity > 99%) followed by coincubation for 72 h in the presence or absence of pHrodo fluorescent dye–labeled ANs at a 1:1 ratio. (C, Upper) After a 72-h incubation, 23% of total CD11c⁻ microglia had ingested ANs (AN⁺), of which 77.4% of these AN⁺ microglia were CD11c⁺. In contrast, the <1% of CD11c⁻ microglia that had not ingested ANs (AN⁻), reflecting 77% of total microglia in culture at 72 h) expressed CD11c. (C, Lower) After incubation with ANs for 72 h, ∼18% of total CD11c⁻ microglia express CD11c, while ∼98% of microglia incubated in the absence of ANs remain CD11c⁻. (D and E) CD11c⁻ microglia isolated from P5 WT mice were incubated in the presence or absence of LPS (10 ng/mL) or Aβ (1 μM) for 24 h followed by analysis of microglial activation markers and CD11c expression. Expression of CD11c was assessed in CD86⁺ or MHC II⁺ activated microglia. Despite up-regulation of CD86 and MHC II in response to LPS and Aβ, CD11c⁻ microglial differentiation was not observed in response to these stimuli. Flow cytometry plots are representative data of three experiments. (F) CD11c⁻ microglia were isolated from P5 WT mice by negative selection with anti-CD11c magnetic beads (purity > 99%) followed by coincubation for 72 h in the presence or absence of pHrodo fluorescent dye–labeled ANs at a 1:1 ratio with or without the αvβ3-integrin inhibitor Cil (10 μM) and/or pan-TAM receptor inhibitor LDC1267 (1 μM). After a 72-h incubation, Cil or LDC1267 reduced AN uptake by CD11c⁻ microglia by ∼50%, and the combination of these two inhibitors further down-regulated AN uptake to background levels. Stimulation by ANs induced CD11c expression by ∼20% of initial CD11c⁻ microglia over 72 h, while CD11c expression was not detectable in the absence of AN stimulation during the same period. Inhibition of AN uptake by Cil or LDC1267 reduced acquisition of a CD11c⁺ phenotype by CD11c⁻ progenitors by about 50 to 75% in each case and inclusion of both inhibitors fully prevented this phenotypic transition (n = 3). ***P < 0.0001, ****P < 0.0001, **P < 0.01, *P < 0.05 by one-way ANOVA with Bonferroni’s multiple-comparisons test; ns, not significant. Data are shown as mean ± SEM.
from OPN-sufficient mice expressed the representative core genes Cd36 and Cd209a at the protein level compared with their CD11c− counterparts in the presence of 5XFAD pathology (SI Appendix, Fig. S6 A and B). Moreover, OPN production contributed to CD11c+ microglial stability, in contrast to strongly reduced stability of CD11c− microglia from age-matched OPN-KO.5XFAD mice (∼40 to 80% reduction) in vitro and ex vivo (SI Appendix, Fig. S6C). The CD11c phenotype was also stably expressed by CD11c+ microglia from 9-mo-old 5XFAD mice in the presence of Aβ42, while OPN deficiency led to 40 to 50% reduction in vitro and in OHSCs, respectively (SI Appendix, Fig. S6D).

Discussion

Microglial expression of the CD11c receptor and production of OPN have been associated with microglial activation during the development of some neuroinflammatory diseases (3, 4, 7) and in response to exogenous stimuli (14). This may be a consequence of microglial activation or, alternatively, a part of the genetic program of a microglial subset that develops at or before birth and persists into late adulthood. Our studies support the latter view, namely that CD11c+ microglia represent a stable subset programmed to produce OPN rather than a transient activation phenotype.

We identify a small (<5%) OPN-producing CD11c+ microglial subset that differentiates from CD11c− progeny of nonspecific microglial activation or inflammatory stimuli that develop in 5XFAD mice, which recapitulate neurodegenerative disease (4–7). After their initial formation in perinatal life, CD11c+ microglia recede to almost undetectable levels in young adult life before reemergence in late adult life to constitute 10 to 15% of total microglia. Both the perinatal CD11c+ microglia and late adult life CD11c+ microglia express a genetic signature that is independent of cellular activation in healthy mice.

Single-cell transcriptomics have described microglial subsets enriched for Itgax (encoding CD11c) at different ages and during development of neurodegenerative disease (4–7), following their description by the Owens group (3), who observed that CD11c+ microglial numbers peaked early after birth (P3 to P5) and were reduced to marginal levels by young adulthood (2 to 3 mo). Our analysis of CD11c+ microglia before birth and during normal aging revealed that CD11c+ microglia that appear during late embryogenesis (E18.5) transiently contract before reemergence and expand into substantial numbers during normal aging.

Although alterations in the microglial gene transcription program after engulfment of apoptotic cells include up-regulation of Itgax gene expression (18, 28), studies of unselected microglial populations have not determined whether this reflects de novo expression of CD11c by CD11c− precursors or expansion of CD11c+ microglia from a smaller population. Analysis of the response of isolated CD11c− precursors to ANs revealed that induction of CD11c protein expression following AN engulfment by neonatal CD11c− precursors was accompanied by OPN expression, suggesting that this phenotype is a direct consequence of AN-induced differentiation. This view is supported by findings that formation of CD11c+ microglia is independent of nonspecific microglial activation, since deliberate activation of CD11c− precursors by various nonspecific stimuli failed to induce expression of the CD11c phenotype. It may be relevant that microglia within neonatal brain regions containing high amounts of apoptotic cells express high levels of Itgax/CD11c and phagocytosis-related genes (3, 6, 29, 30). Moreover, injection of ANs, but not live neurons, Escherichia coli, or zymosan particles, into C57BL/6 mouse brain induces a microglial phenotype that includes up-regulation of Itgax/CD11c (18), suggesting that microglial induction of CD11c is a tightly controlled response to cellular apoptosis. It may also be possible that conversion of adult...
Fig. 2. Stability of CD11c expression by microglia is regulated by OPN. (A) Flow cytometry analysis of OPN expression in CD11c+ versus CD11c− microglia from WT mice at different stages of development and aging (n = 3). OPN production was confined to CD11c+ microglia during early development and normal aging. ****P < 0.0001 by two-way ANOVA with Bonferroni’s multiple-comparisons test. (B) CD11c+ microglia were isolated from P5 WT mice followed by incubation for 72 h with pHrodo fluorescent dye-labeled ANs at a 1:1 ratio. CD11c− microglia that had engulfed ANs differentiated into CD11c+ microglia accompanied by sharply increased expression of OPN (n = 3). ****P < 0.0001 by two-tailed Student’s t test. (C and D) CD11c+ microglia (>95% purity) were isolated from P5 and 9-mo-old WT and OPN-KO mice followed by incubation for 7 d in conventional microglial culture medium (Dulbecco’s modified Eagle’s medium–F12 with 10% FBS + 1% penicillin/streptomycin + 10 ng/mL rmM-CSF). In vitro analysis of CD11c+ microglial stability was evaluated by comparing the percentage of CD11c+ microglia on day 0 with day 7 (percentages were normalized to day 0). CD11c+ microglia of P5 and 9-mo-old WT mice were stable, while OPN-KO CD11c+ microglia displayed a significant loss of stability in P5 and 9-mo-old mice (n = 3). ****P < 0.0001 by two-way ANOVA with Bonferroni’s multiple-comparisons test; ns, not significant. (E and F) CD11c+ microglial stability was assessed using ex vivo OHSCs. Freshly prepared OHSCs were incubated for 24 h with 0.5 mg/mL clodronate liposomes at 35°C to deplete endogenous microglia. Each microglia-free OHSC was reconstituted with 4 × 103 CD11c+ microglia isolated from P5 or 9-mo-old WT or OPN-KO mice (purity >95%). Representative images displaying CD11c+ microglia in hippocampal slices reconstituted with P5 WT or P5 OPN-KO CD11c+ microglia at day 7. CD11c+ microglia from P5 and 9-mo-old WT mice stably expressed CD11c, while OPN deficiency reduced CD11c expression by ∼60% (n = 3 or 4). (Scale bar, 25 μm) ****P < 0.0001, ***P < 0.001 by two-way ANOVA with Bonferroni’s multiple-comparisons test; ns, not significant. (G and H) CD11c+ microglia isolated from 9-mo-old WT and OPN-KO mice were incubated for 7 d in vitro or in OHSCs in the presence of 1 μM synthetic human Aβ1-42 peptide. CD11c+ microglia from 9-mo-old WT mice stably expressed CD11c, while OPN deficiency led to a substantial reduction of the CD11c phenotype in the presence of Aβ1-42 in vitro and in OHSCs, respectively (n = 3). ****P < 0.0001, ***P < 0.01 by two-way ANOVA with Bonferroni’s multiple-comparisons test; ns, not significant. Data are shown as mean ± SEM.
Fig. 3. Definition of an intrinsic genetic program of CD11c+ microglia. (A) Transcriptomic profiling of CD11c+ and CD11c– microglia was analyzed in P5 and 9-mo-old WT mice by RNA-seq. Venn diagrams show the number of genes expressed in P5 and 9-mo-old WT CD11c+ and CD11c– microglia and genes that are exclusively expressed by each microglial subset. CD11c+ microglial unique genes were identified within those genes that showed a fold change and raw counts in the top 0.15% and negatively expressed by their CD11c– counterparts. The threshold of negative expression was defined according to raw counts of genes with no expression, for example Itgax in CD11c– microglia. A similar strategy was used to identify CD11c– microglial unique genes. (B) The core genetic signature of CD11c+ microglia was identified as overlapping unique genes of P5 and 9-mo-old CD11c+ microglia. (C) Heatmap displaying four CD11c+ microglial core genes, including Itgax, Cd209a, Cd209f, and Cd36, in P5 and 9-mo-old CD11c+ microglia compared with CD11c– counterparts (FDR < 0.05). (D and E) Validation of core genes at the protein level. Core genes of CD11c+ microglia were validated at the protein level by flow cytometry analysis. Surface proteins CD36 and CD209a were solely expressed by CD11c+ microglia of P5 and 9-mo-old WT mice compared with CD11c– microglia (n = 3). ****P < 0.0001, **P < 0.01 by two-tailed Student’s t test. (F) CD11c– microglia isolated from P5 WT mice were incubated for 24 h with LPS (10 ng/mL) or Aβ1-42 (1 μM) followed by flow cytometry analysis of CD36 and CD209 expression. Protein expression of these core genes was barely detectable in CD86+CD11c– microglia in response to activating stimuli. Flow cytometry plots are representative of three experiments. (G) In vitro differentiation of CD11c+ microglia. CD11c+ and CD11c– microglia were freshly isolated from P5 WT mice. The CD11c+ microglial phenotype was validated by qPCR analysis of mRNA expression of representative core genes on day 0. Then, CD11c– microglia were incubated for 72 h with ANs to induce CD11c– microglial differentiation into CD11c+ microglia. The core genes were up-regulated in CD11c+ microglia (differentiated) compared with CD11c– microglia (undifferentiated) on day 3. Expression of core genes was normalized into a z score as shown in the heatmap (n = 3). P < 0.05. Data are shown as mean ± SEM.
Microglia later in life can be efficiently promoted by other stimuli including Aβ either alone or as a synergistic mixture of ANs and Aβ.

Expression of the CD11c+ phenotype depends on OPN production, as judged from in vitro analyses and after transfer into microglia-free hippocampal tissues. Moreover, analysis of microglial TNF-α production, as judged from in vitro analyses and after transfer of 9-mo-old WT mice were incubated for 24 h with or without 12.5 μg·mL⁻¹ rmOPN or 10 μM αvβ3 inhibitor (Cil) followed by flow cytometry analysis of the proliferation marker Ki-67 early in development (P5). OPN-s but not OPN-i deficiency decreased Ki-67 expression in CD11c+ microglia (n = 3). **P < 0.0001 vs. OPN-i-KI. ****P < 0.00001 by two-tailed Student’s t test; ns, not significant. (C) CD11c+ microglia expressed high levels of the proliferation marker Ki-67 early in development (P5). OPN-s but not OPN-i deficiency decreased Ki-67 expression in CD11c+ microglia (n = 3). *P < 0.05 by one-way ANOVA with Bonferroni’s multiple-comparisons test; ns, not significant. (D) During aging, CD11c+ microglia showed relatively low levels of proliferative activity and CD11c+ microglial Ki-67 expression was substantially decreased in mice lacking OPN-s (n = 3). *WT vs. OPN-KO; **WT vs. OPN-i-KI. ***P < 0.0001, ****P < 0.00001, *P < 0.05, **P < 0.01 by two-way ANOVA with Bonferroni’s multiple-comparisons test; ns, not significant. (E) Microglia isolated from 9-mo-old WT mice were incubated for 24 h with or without 12.5 μg·mL⁻¹ rmOPN or 10 μM αvβ3 inhibitor (Cil) followed by flow cytometry analysis of the proliferation marker Ki-67. rmOPN enhanced Ki-67 expression in CD11c+αvβ3+ microglia of 9-mo-old WT mice, while this effect was fully abrogated by the αvβ3 inhibitor (n = 3). **P < 0.01, ***P < 0.001 by one-way ANOVA with Bonferroni’s multiple-comparisons test. (F) The expression of tumor necrosis factor α (TNF-α) was barely detectable in CD11c+ microglia of P5 mice, while its expression gradually increased during aging. The age-dependent increase of TNF-α expression in CD11c+ microglia was markedly reduced in WT mice lacking OPN-s but not OPN-i (n = 3). *WT vs. OPN-KO; **WT vs. OPN-i-KI. ****P < 0.0001, *****P < 0.00001, *P < 0.05 by two-way ANOVA with Bonferroni’s multiple-comparisons test; ns, not significant. (G) Microglia isolated from 9-mo-old WT mice were incubated for 24 h with or without 12.5 μg·mL⁻¹ rmOPN or 10 μM αvβ3 inhibitor (Cil) followed by flow cytometry analysis of TNF-α expression. Up-regulation of TNF-α expression induced by rmOPN was fully abolished by the αvβ3 inhibitor in CD11c+αvβ3+ microglia of 9-mo-old WT mice (n = 3). **P < 0.01 by one-way ANOVA with Bonferroni’s multiple-comparisons test. Data are shown as mean ± SEM.
suggesting that CD11c+ microglia are specialized to execute these microglial functions. Indeed, we note that CD11c+ microglia may contribute to neuronal synapse elimination by engulfing synaptic proteins in early development and mediate proinflammatory responses during aging, perhaps enabling elimination of defective or inactive synapses. These functions of the CD11c microglial subset are regulated by OPN; since engulfment of synaptic proteins by neonatal CD11c+ microglia is depressed in the absence of OPN, while the proliferative and proinflammatory responses of CD11c+ microglia in adult life reflect OPN engagement of αβ3-integrin receptors. Analysis of OPN-mutant mice that specifically express OPN isoforms demonstrated that OPN-s, but not OPN-i, is responsible for these OPN-dependent functions. Promotion of inflammatory responses by CD11c+ microglia is reminiscent of the subset of DCs which express high levels of CD11c and carry out OPN-dependent inflammatory responses (33). Our observations that CD11c+ microglia from SFXAD mice express the signature genes at the protein level and the OPN-dependent phenotype noted in healthy mice may allow definition of their potential contribution to disease development.

Taken together, we show that CD11c+ microglia, the sole producer of OPN in the brain, differentiate from CD11c microglia after uptake of ANs at birth. We suggest that this OPN-producing CD11c+ microglial population represents a unique subset according to its stable phenotype and expression of a signature gene set at birth and late adult life that is independent of deliberate activation. The proinflammatory properties of CD11c+ microglia suggest that these cells may contribute to the development of neuroinflammatory diseases including AD and, potentially, ALS and Parkinson’s disease.

Materials and Methods

Mice. WT C57BL/6 (B6) and B6.Cg-Tg(APP1swFlour,PSEN1*M146L*126VS6*799-Vas/Mmjax (SFXAD) mice were obtained from the Jackson Laboratory (MMRRC). Spp1flstop (OPN-KO) and Spp1flstopEllarc (OPN-i-KI) mice were generated by our laboratory as previously described (34). OPN-KO/SFXAD were bred by crossing Spp1flstop mice with SFXAD mice. All the mice were housed in pathogen-free conditions. All experiments were performed in compliance with federal laws and institutional guidelines as approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

Microglial Isolation.

Isolation of microglia by magnetic-activated cell sorting. Adult mice were anesthetized with isoflurane and tranversally perfused with ice-cold phosphate-buffered saline (PBS) before brains were removed and minced by scalpels. After addition to enzymatic solution and induction of enzymatic dissociation using collagenase (300 U/ml Worthington) or papain (20 U/ml Worthington) and DNase I (60 U/ml Worthington) before a 30% Percoll gradient was performed to remove myelins, and pelletted cells were resuspended in magnetic-activated cell sorting (MACS) buffer (PBS, pH 7.2, 2 mM ethylenediaminetetraacetic acid [EDTA], and 0.5% bovine serum albumin [BSA]). Total microglia were obtained by magnetic isolation using CD11b microbeads (Milteny). For isolation of CD11c+ and CD11c microglia, single-cell suspensions were incubated with CD11c microbeads (Milteny) and cells magnetically bound to columns using MACS were extensively washed before the CD11c fraction was eluted after lifting the magnetic field. The unbound fraction was then labeled with CD11b microbeads (Milteny) and separated using MACS isolation, and CD11c+ cells that were bound to the column were eluted. MACS buffer was used according to the manufacturer’s suggested protocol. This standard method was used to isolate microglia for all of the experiments unless otherwise noted.

Microglial isolation and FACS for RNA-seq analysis. Single-cell suspensions were prepared as described (6). Briefly, mice were anesthetized with isoflurane and tranversally perfused before brains were quickly dissected and minced using a scalpel on ice followed by Dounce homogenization in ice-cold Hank’s balanced salt solution (HBSS) – 20 times. All tools used were prechilled and all isolation steps were carried out on ice to minimize microglial activation. Cell suspensions were transferred to prechilled 50-μl tubes and passed through a 70-μm cell strainer followed by transfer into a prechilled 15-μl tube and spun down at 500 × g for 5 min at 4 °C. Debris and myelin were then removed using a modified cold Percoll gradient and cell pellets were resuspended in 10 μl of ice-cold 40% Percoll (Sigma), diluted, and then spun for 30 min at 500 × g x 30. This approach yielded a microglial pellet at the bottom of the 15-μl tube while Percoll and myelin were removed by vacuum suction.

The cell pellet was washed with 10 μl of ice-cold HBSS and spun again for 5 min at 500 × g at 4 °C. All samples were then resuspended in ice-cold FACS buffer (0.5% BSA, 1 mM EDTA, in 1× PBS) for staining. Ghost Dye violet 510 (1:1,000; Tonbo Biosciences) was used to exclude dead cells. Fc receptors were blocked using anti-CD16/CD32 antibody (1:100; BD Biosciences) to avoid non-specific staining. Single-cell suspensions were then stained with anti-CD11b (1:100), anti-CD45 (1:100), and anti-CD11c (1:50) antibodies (BioLegend) for 20 min on ice before samples were washed with ice-cold FACS buffer and spun down for 5 min at 500 g. Cell pellets were resuspended in 5 mL of ice-cold FACS buffer before sorting on a BD FACSAria II using the 70-μm nozzle with purity mode at ~10,000 events per second. After sorting, each sample was spun down and cell pellets were immediately stored at −80 °C until further processing.

For P5 mice, three biological replicates of CD11c+ microglia and CD11c+ microglia were sequenced. Samples were pooled from 58 mice. For 9-month-old WT mice, two replicates of CD11c+ microglia and CD11c+ microglia were sequenced. Samples were pooled from 44 mice (22 male and 22 female).

AN Induction and Labeling for CD11c Microglial Differentiation. Primary mouse neurons were prepared from B6 embryos at E16.5 to E17.5. Cerebral hemispheres were isolated and freed from meninges before tissue digestion with 0.25% trypsin in HBSS at 15 min at 37 °C followed by trypan blue staining. Total apoptotic cell numbers were determined using trypan blue staining.

Flow Cytometry.

Microglial staining for flow cytometry analysis. Microglia were stained with Ghost Dye violet 510 (1:1,000; Tonbo Biosciences) to exclude dead cells followed by Fc receptor blocking using an anti-CD16/CD32 antibody (1:100; BD Biosciences) to avoid nonspecific staining. Appropriate microglial surface markers were used for staining, including anti-CD11b (1:100; BioLegend), anti-CD45 (1:100; BioLegend), anti-CD11c (1:50; BioLegend), anti-CD36 (1:100; BioLegend), anti-CD209a (1:100; BioLegend), anti-α-β integrin (1:50; BioLegend), anti-β-2 integrin (1:50; BioLegend), anti-CD86 (1:100; BioLegend), anti-MHC II (1:100), anti-MHC II (1:50; BioLegend), followed by intracellular staining and permeabilization with intracellular staining with anti-OPN (1:100; R&D Systems) and anti-TH-1 (1:50; BioLegend) and intranuclear staining with anti-Ki-67 (1:100; BioLegend).

Validation of microglial OPN expression by flow cytometry analysis. Microglial OPN expression was validated in 9-month-old WT mice using conventional intracellular staining protocols. Microglia were fixed and permeabilized with Intracellular Fixation & Permeabilization Buffer (eBioscience) followed by incubation with a phycoerythrin (PE)-conjugated anti-OPN antibody (1:10; IC808; R&D Systems) at 4 °C for 30 min. An isotype control (1:10; PE-conjugated goat immunoglobulin G [lgG] and OPN-KO microglia were used as a negative control. Microglia that selectively express the intracellular isomorph of OPN (OPN-i-KI) were used as a positive control.

Validation of microglial CD11c expression by flow cytometry analysis. Brain single-cell suspensions from 9-month-old WT mice were stained with Ghost Dye violet 510 (1:1,000; Tonbo Biosciences) and anti-CD16/CD32 antibody (1:100; BD Biosciences), followed by anti-CD11b (1:100; BioLegend), anti-CD45 (1:100; BioLegend), anti-CD11c (1:50; BioLegend), anti-TMEM119 (1:200; Abcam), and anti-CCR2 (1:100; BioLegend). CD11b+ cells were gated from singlecell live samples followed by subsequent gating of CD11b+CD45+ as microglia and CD11b+CD45− as macrophages. To distinguish microglia and macrophages, the CCR2 marker expressed by blood-derived macrophages but not by microglia was used to distinguish these populations. The non-specific marker Tmem119 (17) was also included. The CD11b+CD45+ cells that express CCR2 but not Tmem119 were confirmed as macrophages, while the CD11b+CD45− cells that express Tmem119 but not express CCR2 were confirmed as microglia. FMO negative controls were included to confirm
the specificity of CD11c staining in CD11b+CD45+ microglial populations. Brain CD45 cells that mainly contain nonimmune cells (e.g., neurons, astrocytes, and oligodendrocytes) that do not express CD11c were also included as negative controls to further validate the specificity of this strategy.

**Flow cytometry detection of microglial engulfment of synaptic protein.** After perfusion, mouse brains were harvested and dissected followed by mey-in removal by centrifugation on a 30% Percoll gradient. Brain pellets were sequentially stained with Ghost Dye violet 510 (1:1,000; Tonbo Biosciences) followed by incubation with the microglial surface marker anti-CD11b, anti-CD45, and anti-CD11c for 30 min. Subsequently, stained samples were fixed and permeabilized using Intracelar Fixation & Permeabilization Buffer (eBioscience). Intracellular staining was performed for the preysmatic marker anti-synaptophysin (1:100; Invitrogen) or postsynaptmic marker PSD95 (1:100; Invitrogen) followed by staining with Alexa Fluor 488–donkey anti-mouse IgG (H+L) secondary antibody (1:300; Invitrogen). Samples were acquired on a CytoFLEX (Beckman Coulter) flow cytometer followed by analysis with FlowJo v10 (Tree Star).

**OHSs.** OHSs were prepared as described (35). Briefly, hippocampal slices were prepared from newborn (P3 to P5) C57BL/6 mice to a thickness of 350 μm before incubation at 35 °C in 5% CO2. Microglia were depleted from freshly prepared slice cultures using clodronate liposomes (Formu-MADE). In brief, OHSs were incubated with 0.5 mg/ml clodronate liposomes for 24 h at 35 °C. Subsequently, OHSs were rinsed with warm PBS before replacement of medium (50% modiﬁed DMEM, 25% FCS, 100 mg/mL streptomycin, and 4.5 mg/mL glucose). Microglia-depleted OHSs were maintained for 7 d before experimentation. CD11c+ microglia were acutely isolated from P5 or 9-mo-old WT and OPN-KO mice or 9-mo-old WT and OPN-KO mice and perfused with a SMARTana v4 Ultra Low Input Kit for Sequencing full-length cDNA synthesis and amplification (Clontech) and an Illumina Nextera XT library for sequencing library preparation. Briefly, cDNA was fragmented and adapter was added was transposase, followed by limited-cycle PCR to enrich and add index to the cDNA fragments. The final library was assessed with an Agilent TapeStation and quantified using a Qubit 2.0 fluorometer (Invitrogen) as well as by qPCR (KAPA Biosystems).

**Ultra-low-input strategy.** Due to the very limited cell numbers obtained from adult mice, CD11c+ and CD11c– microglial samples of 9-mo-old WT mice were used with a SMARTana Ultra Low Input Kit for Sequencing for full-length cDNA synthesis and amplification (Clontech) and an Illumina Nextera XT library for sequencing library preparation. Briefly, cDNA was fragmented and adapter was added was transposase, followed by limited-cycle PCR to enrich and add index to the cDNA fragments. The final library was assessed with an Agilent TapeStation and quantified using a Qubit 2.0 fluorometer (Invitrogen) as well as by qPCR (KAPA Biosystems).

**HiSeq sequencing.** The sequencing libraries were clustered on flowcell lanes before the flowcell was loaded onto an Illumina HiSeq instrument (4000 or equivalent) per the manufacturer’s instructions. Samples were sequenced using a 2 × 150-bp paired-end configuration, and image analysis and base calling were conducted by HiSeq Control Software. Raw sequence data (`.bcl` files) generated from Illumina HiSeq were converted into fastq flexible and demultiplexed using Illumina’s bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

**RNA-seq data analysis.** Mapping and gene counting were performed by GENEWIZ. After reviewing the quality of the raw data, sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v0.36. The trimmed reads were mapped to the Mus musculus reference genome (ENSEMBL) using STAR aligner v2.5.2b, a splice aligner that detects and incorporates splice junctions to align the entire read sequences. BAM files were generated, and unique gene hit counts were calculated using Count (Subread package v1.5.2). Only unique reads that fell within exon regions were counted. Differential expression was considered significant with a false discovery rate (FDR)–adjusted P value < 0.05.

**In vitro Synaptosome Engulfment Assay.** **Synaptosome isolation and labeling.** Synaptosomes were isolated from WT mice using Syn-Per Synaptic Protein Extraction Reagent (Thermo Scientific), per the manufacturer’s instructions. For pHrodo labeling, dissolved pHrodo IFL green (Life Technologies) was incubated with synaptosomes on a shaker in PBS for 1 h at room temperature protected from light at the ratio of 20 μg pHrodo per 1 mg synaptosomes. Unconjugated pHrodo was removed by washing with PBS before pHrodo-conjugated synaptosomes were resuspended in PBS with 5% dimethyl sulfoxide, aliquoted, and stored at −80 °C until use. **Microglial engulfment of synaptosomes.** Microglia isolated from P5 and 9-mo-old WT, OPN-KO, and OPN-KO mice were incubated with 136 μg pHrodo green–labeled synaptosomes per 1 × 106 cells for 1 h followed by staining for 30 min with Ghost Dye violet 510 (1:1,000, Tonbo Biosciences) and anti-CD11c (1:50, BioLegend). CD11c+ microglial engulfment of synaptosomes was assessed by flow cytometry analysis.

**Quantification and Statistical Analysis.** Data in figures are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism v9.0. Quantification of fluorescence microscopy images was performed using Image J. Statistical analysis to compare the mean values for multiple groups was performed using GraphPad Prism by one- or two-way ANOVA with Bonferroni’s multiple-comparisons test. Comparisons between two groups were analyzed using two-tailed Student’s t test. P value < 0.05 was considered statistically significant. All of the statistical details of experiments, including the statistical tests used and exact value of sample sizes, can be found in the figure legends.

**Data Availability.** The RNA-seq data reported in this article have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through GEO Series accession no. GSE190713. All study data are included in the article and/or SI Appendix.
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