Activated microglia enhance neurogenesis via trypsinogen secretion

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White matter neurons in multiple sclerosis brains are destroyed during demyelination and then replaced in some chronic multiple sclerosis lesions that exhibit a morphologically distinct population of activated microglia [Chang A, et al. (2008) *Brain* 131(Pt 9):2366–2375]. Here we investigated whether activated microglia secrete factors that promote the generation of neurons from white matter cells. Adult rat brain microglia (resting or activated with lipopolysaccharide) were isolated by flow cytometry and cocultured with neonatal rat optic nerve cells in separate but media-connected chambers. Optic nerve cells cocultured with activated microglia showed a significant increase in the number of cells of neuronal phenotype, identified by neuron-specific class III beta-tubulin (TUJ-1) labeling, compared with cultures with resting microglia. To investigate the possible source of the TUJ-1–positive cells, A2B5-positive oligodendrocyte progenitor cells and A2B5-negative cells were isolated and cocultured with resting and activated microglia. Significantly more TUJ-1–positive cells were generated from A2B5-negative cells (~70%) than from A2B5-positive cells (~30%). Mass spectrometry analysis of microglia culture media identified protease serine 2 (PRSS2) as a factor secreted by activated, but not resting, microglia. When added to optic nerve cultures, PRSS2 significantly increased neurogenesis, whereas the serine protease inhibitor, secretory leukocyte protease inhibitor, decreased activated microglia-induced neurogenesis. Collectively our data provide evidence that activated microglia increase neurogenesis through secretion of PRSS2.

serine protease | GFAP2

Neurogenesis in the adult mammalian brain can be modulated by many factors, including environmental enrichment, learning, stress, aging, and injury (1). Studies also support neurogenesis in human neurodegenerative diseases, including the hippocampus of Alzheimer's disease brains and the subependymal layer of Huntington disease brains (2). Subcortical white matter contains a population of neurons that originate from cells that form the subcortical plate during embryonic brain development (3, 4). These neurons have an interneuron phenotype and participate in the regulation of vascular tone (5). We have described neurogenesis in a subset of chronic white matter lesions from postmortem multiple sclerosis (MS) brains based upon increased densities of mature and immature neurons compared with control brains (6). Although the source of new neurons in lesions of MS is unknown, studies in the rodent (7, 8) and human brain (9) have suggested that oligodendrocyte progenitor cells (OPCs) can generate interneurons.

Microglia, the resident immune cells of the central nervous system, have important roles in innate immunity and inflammatory neuropathology (10, 11). Chronic white matter MS lesions with increased neuronal densities contained a population of activated microglia with a unique morphology, suggesting that microglia may participate in the generation of white matter neurons, possibly by the secretion of soluble factors (6). Whereas some studies have reported that activated microglia can have a detrimental effect on neurogenesis (12–15), others have shown that activated microglia enhance cell proliferation and differentiation (16–19). These differential effects may reflect different microglial activation states that are related to either proinflammatory destructive phenotypes or anti-inflammatory tissue repair phenotypes.

To directly address the role of activated microglia in neurogenesis, we investigated whether factors secreted by activated microglia increase the generation of neurons from white matter cells. Activated and resting microglia were isolated by flow cytometry and cocultured with dissociated neonatal optic nerve cells (ONCs). In a subset of our studies, we separate OPCs and the remainder of the dissociated ONCs. Coculturing of activated, but not resting, microglia with neonatal white matter cells increased neurogenesis in vitro. Furthermore, our studies support the possibility that activated microglia increase neurogenesis by secreting protease serine 2 (PRSS2), an inactive form of trypsin.

Results

Activated Microglia Increase Neuron-Specific Class III Beta-Tubulin–Positive Cells. Cells of monocyte origin including microglia, monocytes, and macrophages express CD11b. CD11b-positive activated and resting microglia can be separated by flow cytometry based on relative levels of CD45 expression. Activated microglia were isolated from rats that received four daily i.p. injections of LPS (1 mg/kg body weight) (20). This paradigm globally activates microglia that display an M2 phenotype (20), which is considered anti-inflammatory and tissue-repairing (21). Resting microglia were obtained from rats that received four daily i.p. injections of PBS. Resting microglia were CD11b-positive, CD45-low (Fig. 1*A*), whereas activated microglia were CD11b-positive, CD45-intermediate (Fig. 1*B*). As additional controls, macroglia (CD11b-negative, CD45-negative) were isolated from LPS- and PBS-injected rats.

To investigate the effects of innate immune cell subpopulations on the generation of white matter neurons, we cocultured neonatal rat ONCs with resting microglia, LPS-activated microglia, control macroglia, and LPS-activated macroglia. Characterized cells in neonatal optic nerves include OPCs, immature and mature astrocytes, endothelial cells, and vessel-associated pericytes. Following dissociation and plating on poly-L-lysine (PLL)/laminin-coated coverslips maintained in media containing 1% serum, the majority of cells are OPCs and immature and mature astrocytes. Endothelial cells do not adhere to the PLL/laminin-coated coverslips (22–25) and both endothelial cells and pericytes require high levels (>10%) of serum (22, 23, 26) or growth factors (25) to survive in vitro. The dissociated neonatal optic nerve cultures do not contain microglia (27) (Fig. S1).

The ONCs and macro/microglia were cultured for 7 d in separate cell culture chambers (inserts of 20-nm pore size) that shared medium but prevented cell–cell contact (Fig. 1*C*). As an

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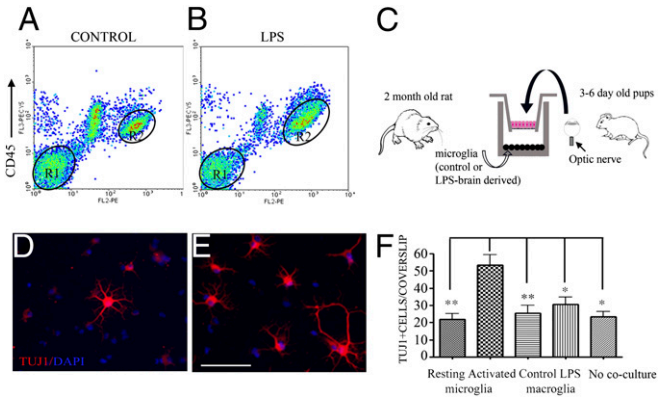

Fig. 1. Secreted factors from activated microglia increase the number of TUJ-1–positive neurons generated from neonatal ONC cultures. (*A* and *B*) Density-plot graphs of FACS-sorted cells. R1 cell population, CD45-negative/CD11-negative macroglial cells (oligodendrocytes OPCs and astrocytes); R2, the CD45-positive/CD11b-positive microglia. The R2 cell population (*A*) shows less granularity and the expression of CD45-positive is lower (resting microglia) compared with the R2 population in *B* (activated microglia). (*C*) Schematic of coculture system. FACS-sorted microglial and macroglial cells were cocultured with dissociated ONCs placed on inserts from rat pups 3 to 6 d of age. (*D*–*F*). Number of TUJ-1–postive neurons in ONC cultures. Coculturing with activated microglia (*E*) induced a significantly greater number of TUJ-1–positive neurons compared with resting microglia (*D*), either macroglia group, or no coculture. Quantification is shown in *F*. $*P < 0.05$. (Scale bar: 50 μm.)

additional control, ONCs were cultured alone (no coculture). A one-way ANOVA showed a significant overall difference in the number of neurons generated among the five groups tested ($P < 0.0001$). Based on Tukey's post hoc analysis, ONCs cocultured with activated microglia showed a significant increase in neuron-specific class III beta-tubulin (TUJ-1)–positive cells (Fig. 1 *E* and *F*) compared with ONCs cocultured with resting microglia ($P < 0.01$, Fig. 1 *D* and *F*), control macroglia ($P < 0.005$, Fig. 1*F*), LPS macroglia ($P < 0.05$, Fig. 1*F*), or ONCs with no coculture ($P < 0.05$, Fig. 1*F*). The total number of cells was not different (Table S1) between conditions, suggesting that the increase of TUJ-1–positive cells reflected neurogenesis as a result of coculturing the ONCs with activated microglia and not differential cell survival or proliferation. These studies establish that factors secreted by activated, but not resting, microglia enhance neurogenesis in neonatal ONC cultures.

OPCs Are Not the Major Source of Neurons in the ONC Cultures. ONCs can generate neurons in vitro (28, 29), and previous studies have implicated OPCs as a source of neurons in ONC cultures (29). We isolated A2B5-positive cells (oligodendrocyte progenitor cells) by immunopanning and cocultured A2B5-positive OPCs and A2B5-negative macroglial cells (GFAP-positive cells) with activated or resting microglia for 7 d and then assayed for the expression of neuronal (TUJ-1) and glial (GFAP, A2B5, and NG2) markers. The number of TUJ-1–positive cells generated from A2B5-positive OPCs was ∼30% of the number of TUJ-1 cells derived from total ONCs (Table S2). Coculturing with activated microglia significantly increased the number of TUJ-1–positive cells generated by A2B5-positive OPCs. This increase was also ∼30% of the increase obtained when ONCs were cocultured with activated microglia (Tables S1 and S2 and Figs. 1*F* and 2*C*). The number of TUJ-1–positive cells generated from total ONCs (Fig. 1*F* and Table S1) was significantly greater than the number of TUJ-1–positive neurons generated from A2B5-positive cells (Fig. 2*C* and Table S2) when cocultured with resting microglia ($P < 0.01$), activated microglia ($P = 0.0001$), control macroglial cells ($P < 0.01$), LPS-treated macroglial cells ($P < 0.001$), or without coculture ($P < 0.01$). The total number of DAPI-positive cells was similar in all culture conditions (Tables S1–S3). These results suggest that A2B5-positive OPCs are not the major cellular source of TUJ-1–positive neurons generated from ONCs.

To further explore the source of TUJ-1–positive neurons in ONC cultures, we quantified the number of TUJ-1–positive cells arising from A2B5-negative ONCs (Fig. 2*D* and Table S3). In general, A2B5-negative cells proportionally generated more TUJ-1–positive cells than A2B5-positive OPCs (compare Tables S2 and S3 and Fig. 2 *C* and *D*), but fewer TUJ-1–positive cells than the total ONCs (compare Tables S1 and S3 and Figs. 3*D* and 1*F*). A2B5-negative macroglia produced ∼70% of the TUJ-1–positive cells that were generated from ONC cultures. Like the A2B5-positive population, A2B5-negative macroglia produced significantly more TUJ-1–positive cells when cocultured with activated microglia than when cocultured with resting microglia ($P < 0.05$) or no cells ($P < 0.05$) (Table S3 and Fig. 2*D*). Not all ONC populations generated neurons. O4 is a lipid antigen expressed by immature and mature oligodendrocytes (30), and immunopanned O4-positive cells did not generate TUJ-1–positive cells even when cocultured with activated or resting microglia. Collectively, our results show that ∼30% of neonatal optic nerve TUJ-1–positive cells are derived from A2B5-positive OPCs and ∼70% from A2B5-negative cells. Secreted factors from activated, but not resting, microglia can significantly increase the number of TUJ-1–positive cells generated by both cell populations (Fig. 2 *C* and *D*). When A2B5-negative cultures were cocultured with activated microglia, occasional TUJ-1–

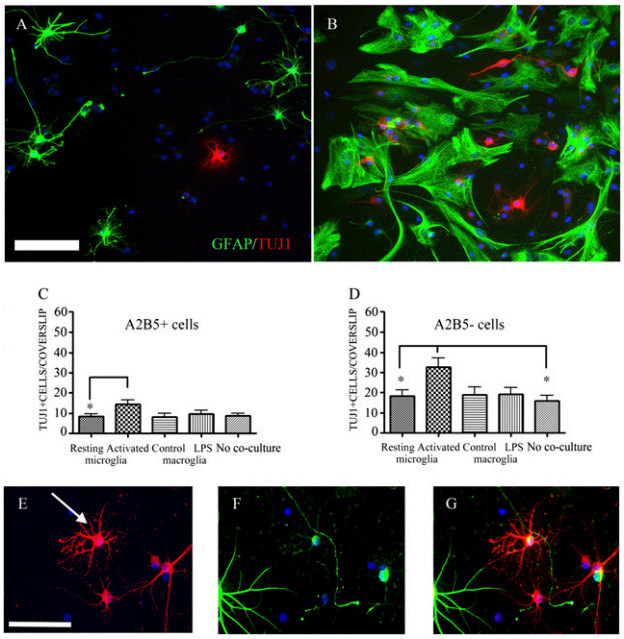

Fig. 2. Oligodendrocyte progenitor cells are not the major source of TUJ-1–positive neurons. (*A*–*D*) A2B5-positive and A2B5-negative ONCs were cocultured with resting microglia, activated microglia, control macroglia, LPS macroglia, or no cells. A2B5-positive cultures (*A* and *C*) generated fewer TUJ-1–positive neurons than did A2B5-negative cultures (*B* and *D*). Compared with other conditions, activated microglia significantly increased the density of TUJ-1–positive neurons in both A2B5-positive (*C*) and A2B5-negative (*D*) ONC cultures. The number of TUJ-1–positive neurons generated from A2B5-negative plus A2B5-positive ONC cultures was similar to the number of TUJ-1–positive neurons generated from whole ONCs (Tables S1–S3). In A2B5-negative cultures, occasional TUJ-1–positive cells were weakly positive for GFAP (*E*–*G*), supporting the possibility that some TUJ-1–positive cells are derived from a GFAP-positive cell. $*P < 0.05$. (Scale bars: 100 μm.)

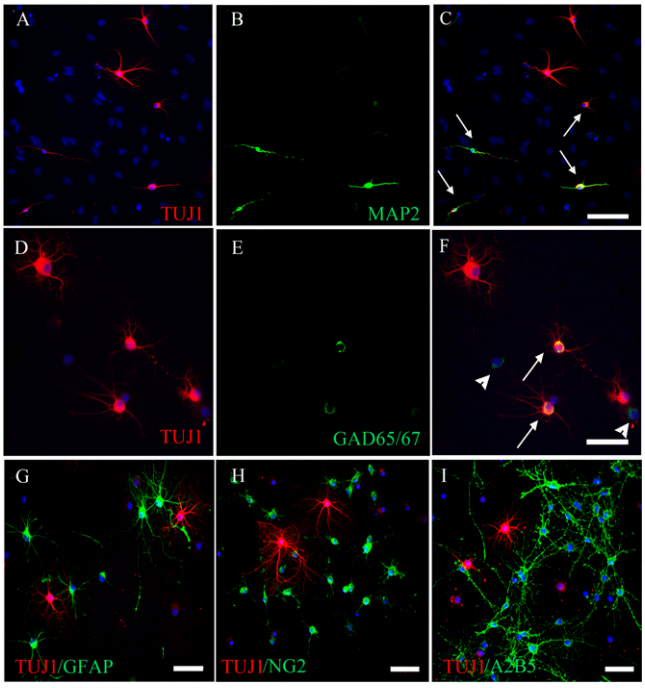

Fig. 3. TUJ-1–positive cells express mature neuronal markers. (*A–C*) ONCs were cocultured with activated microglia for 7 d and stained for neuronal markers. TUJ-1–positive cells (*A* and *D*, red) colabel with microtubule-associated protein 2 (*B*, green), a marker for mature neurons and glutamic acid decarboxylase (GAD) (*E*, green), a marker for GABAergic interneurons. *C* and *F* show merged images of TUJ-1 and MAP2 (*C*, arrows) and GAD (*F*, arrows). TUJ-1–positive neurons (*G–I*, red) are not intensely labeled by astrocytic (GFAP, *G*, green), or OPC (NG2, *H*, green or A2B5, *I*, green) markers. (Scale bars: *A–C*, 100 μm; *D–I*, 50 μm.)

positive neurons were weakly positive for GFAP (Fig. 2 *E–G*). A BrdU pulse from day 3 to day 7 in vitro labeled fibrous astrocytes but did not label TUJ-1–positive cells (Fig. S2 *A* and *B*). Taken together our data support the possibility that the neurons generated in A2B5-negative ONCs are derived from GFAP-positive cells and that proliferation of this cell does not immediately precede neuronal differentiation.

TUJ-1–positive Cells Generated from Dissociated ONCs Have an Interneuron Phenotype. To further characterize the phenotype of TUJ-1–positive cells, we cocultured ONCs and activated microglia for 7 d and assayed for additional neuronal and glial biochemical characteristics markers. Greater than 75% of the TUJ-1–positive cells expressed microtubule-associated protein 2 (MAP2), a marker for mature neurons (Fig. 3 *A–C*) and 65% expressed glutamate decarboxylase 65/67 (GAD65/67), a marker for GABAergic interneurons (arrows, Fig. 3 *D–F*). There were also occasional cells that were GAD65/67-positive and TUJ-1–negative (arrowheads, Fig. 3*F*). As described previously (31, 32), these TUJ-1–negative, GAD 65/67-positive cells are likely to be a subpopulation of astrocytes. The majority of TUJ-1–positive cells were negative for the astrocyte marker GFAP (Fig. 3*G*) and none expressed the OPC markers NG2 (Fig. 3*H*) and A2B5 (Fig. 3*I*). These results indicate that the TUJ-1–positive cells generated from ONCs express markers of mature neurons and exhibit characteristics of interneurons.

Microglial Modulators of TUJ-1 Expression. We used gene profiling and mass spectrometry to identify factors secreted by activated microglia that may induce neurogenesis. For microarray analyses, total mRNA was extracted from resting ($CD45^{low}$/CD11b-positive) and activated ($CD45^{intermediate}$/CD11b-positive) microglia that were isolated from five LPS- and five PBS-treated rats, respectively, by FACS sorting. Quantitative analysis identified gene transcripts that encoded secreted proteins, which were significantly (>1.5 fold; $P < 0.05$) different between resting and activated microglia (Table S4). Up-regulated mRNAs in activated microglia encoded IL1b and CXCL2 (fourfold increase), both expressed by immune cells, and CXCL13 (25-fold), a chemokine that is expressed by microglia in demyelinating lesions obtained from postmortem MS brains (33). mRNA encoding secretory leukocyte protease inhibitor (SLPI) was reduced sixfold in activated microglia. SLPI is a serine protease inhibitor that inhibits a wide range of proteases, including neutrophil elastase, cathepsin G, chymotrypsin, and trypsin (34, 35). Quantitative RT-PCR (qRT-PCR) analysis also detected a significant decrease ($P < 0.02$) in SLPI mRNA levels in activated microglia compared with resting microglia, thereby validating the mRNA changes generated from microarray analyses (Fig. S3 and Table S4). Analysis of media from activated and resting microglia cultured for 8 d by mass spectrometry identified PRSS2 (commonly termed trypsinogen) in activated microglia conditioned medium but not in resting microglia conditioned medium (Table S5). It should be noted that although all samples were digested with modified trypsin that resists autolysis, trypsinogen was only detected in media from activated microglia, indicating that our data are not biased by trypsin treatment during the mass spectrometry analysis.

Gene profiling and mass spectrometry experiments suggested that activated microglia increased secretion of PRSS2, interleukin 1β (IL1β), chemokine (C-X-C motif) ligand 2 (CXCL2), or C-X-C motif chemokine 13 (CXCL13). To test whether these proteins facilitate neurogenesis in optic nerve cultures, they were added to cultures of ONCs cocultured with and without resting microglia at concentrations of 0.5 and 1 μg/mL. In contrast, expression of the serine protease inhibitor SLPI was significantly decreased in activated microglia. Therefore, we added SLPI to activated microglia–optic nerve cocultures to test whether it would reduce the induction of TUJ-1–positive neurons by inactivating the action of microglia-secreted trypsinogen. These ONCs were cultured for 7 d, labeled with antibodies to TUJ-1, and the number of neurons was determined to evaluate the neurogenic potential of each added factor. PRSS2 produced a statistically significant increase in TUJ-1–positive cells when added to ONCs cocultured with resting microglia (one-way ANOVA, $P < 0.05$) or ONCs in the absence of cocultured cells (one-way ANOVA, $P < 0.01$). The addition of 1 μg of PRSS2 to cocultures with resting microglia significantly increased the number of TUJ-1–positive cells from ONCs (Tukey's post hoc analysis, $P < 0.05$; Fig. 4*A*). We observed similar results in cultures with ONCs alone (Tukey's post hoc analysis, $P < 0.01$; Fig. 4*B*). There was no change in neurogenic potential after the addition of any of the other proteins to ONC cultures with resting microglia or ONCs alone regardless of the dose administered (CXCL13, $P = 0.78$; IL1β, $P = 0.37$; and CXCL2, $P = 0.41$). Addition of the serine protease inhibitor SLPI to activated microglia–ONC cocultures inhibited the activated microglial-induced neurogenesis; the number of TUJ-1–positive cells was comparable to that of resting microglial–ONC cocultures or ONC cells alone ($P = 0.6$). Addition of SLPI in ONCs alone did not increase TUJ-1–positive cell production ($P = 0.576$). These data support the hypothesis that secretion of PRSS2 by activated microglia enhances neurogenesis in dissociated neonatal ONCs in vitro and that the serine protease inhibitor, SLPI, inhibits this neurogenic affect.

Discussion

In the present study we used dissociated neonatal rat ONCs as a model to study neurogenesis from white matter neural cells in vitro. Activated or resting microglia were cocultured with ONCs in chambers that prevented physical contact between optic

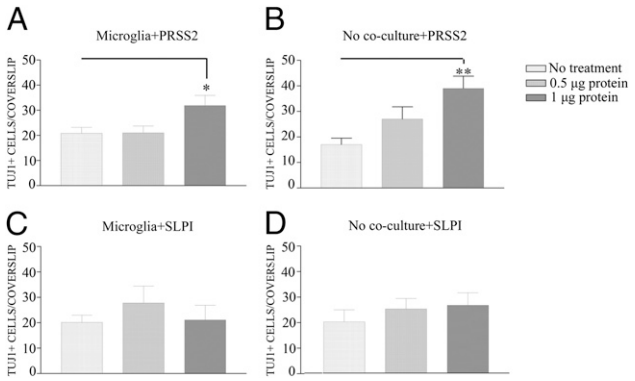

Fig. 4. PRSS2 increases TUJ-1–positive cells when added to cocultures of ONCs with resting microglia and blank media. (*A*) When PRSS2 was added to optic nerve and resting microglia cocultures, the number of TUJ-1–positive cells was significantly increased (one-way ANOVA, $P = 0.038$) compared with the number of neurons generated in cultures containing ONCs and resting microglia without PRSS2 (Tukey's post hoc analysis, $P < 0.05$). (*B*) PRSS2 had a neurogenic effect when added to ONCs without other cocultured cells (one-way ANOVA, $P = 0.004$). When added to ONC cultures, PRSS2 (1 μg) significantly increased the number of TUJ-1–positive cells generated compared with ONCs cultured without PRSS2 (Tukey's post hoc analysis, $P < 0.01$). (*C*) Addition of SLPI to activated microglia abolished the activated microglia-induced neurogenesis and no differences were detected in the number of TUJ-1–positive cells compared with resting microglia (first column from left on graph, one-way ANOVA, $P = 0.6$). (*D*) When SLPI is added to ONCs without other cocultured cells it does not increase the number of TUJ-1–positive cells (one-way ANOVA, $P = 0.576$). $^*P < 0.05$, $^{**}P < 0.01$.

nerves and microglia cells. We isolated highly enriched populations of activated and resting microglia directly from the brain using flow cytometry and well-established surface markers (36, 37). Microglia, the resident immune cells in the brain, play crucial roles in innate immunity and the pathogenesis of neurodegenerative diseases including MS (11), Alzheimer's disease (38, 39), and traumatic brain injury (40). The role of activated microglia during neurogenesis has been debated, with different studies supporting decreased (12, 13) and increased (16–18, 41) neurogenesis. Our data indicate that products secreted by activated microglia significantly increased the number of cells displaying a neuronal phenotype identified by TUJ-1 staining. In addition, most of these neurons were MAP2-positive (a marker of neuronal maturation) and expressed GAD65/67, a marker for inhibitory interneurons. A2B5-negative cells seem to be the main source (~70%) of TUJ-1–positive cells; A2B5-positive OPCs generated ~30% of the TUJ-1–positive neurons. Microarray comparisons of activated and resting microglia and mass spectrometry analyses of culture medium obtained from activated and resting microglia implicated PRSS2, an inactive precursor of trypsin, as an inducer of the TUJ-1 phenotype. When added to ONCs cocultured with resting microglia or ONCs alone, PRSS2 significantly increased TUJ-1–positive cells.

Although immature and mature rodent optic nerves do not contain neuronal perikarya, previous studies have established that neonatal ONCs can generate neurons in vitro (28, 29). It is well established that the in vivo and in vitro potential of individual progenitor cells can vary. For instance, OPCs, identified by A2B5 or NG2 antibodies, produce both astrocytes and oligodendrocytes in vitro (42, 43) but only oligodendrocytes in vivo (44). Our data are consistent with previous in vitro studies that have shown that A2B5-positive OPCs produce stellate, or type-2, astrocytes and that A2B5-negative neonatal optic nerve cultures produce type-1, or fibrous, astrocytes (45). Contrary to expectations, our studies did not support OPCs as the major source of TUJ-1–positive cells in optic nerve cultures. A2B5-negative optic nerve cultures produced significantly greater numbers of TUJ-1–positive neurons than did A2B5-positive cell cultures. Our studies support the possibility that the major precursor of TUJ-1–positive cells is a GFAP-positive cell, because TUJ-1–positive cells were occasionally weakly positive for GFAP. We assume this is a transient expression of a precursor-specific molecule that occurs during early stages of TUJ-1 cell differentiation. There is precedent for this: Transient expression of A2B5 can be detected on astrocytes as they differentiate from A2B5-positive cells in vitro (46). Former studies have identified GFAP-positive neuronal precursor cell populations that have stem cell properties. GFAP-positive cells in the adult subventricular zone are a primary neuronal precursor in vivo (47) and a source of new interneurons in the olfactory bulb (48), whereas GFAP-expressing cells in the hippocampal subgranular layer act as neuronal precursors in the adult brain (49). Although the precise nature of the neuronal precursor in A2B5-negative neonatal optic nerve cultures remains to be determined, GFAP-positive cells may play the role of neuronal precursors. Molecules secreted from activated, but not resting, microglia significantly increased TUJ-1–positive cells generated from both OPC-negative and OPC-positive cell populations, making it likely that the factor secreted by activated microglia acts directly on both cell populations.

To investigate the nature of the microglial-secreted factor that enhances neurogenesis, we analyzed media from resting and activated microglia by mass spectrometry. These studies identified PRSS2 as a molecule secreted by activated, but not resting, microglia. PRSS2 belongs to the serine protease superfamily that includes kallikrein and activated protein C, which participate in cell proliferation, migration, and differentiation (37, 50, 51). PRSS2 is a trypsinogen, a precursor form of trypsin, which has been reported to be neuroprotective, possibly via activation of proteinase-activated receptor-2 (PAR2) (52). PAR2 is expressed in mammalian neurons (53, 54), astrocytes (52, 55), and microglia (56). Trypsin activates PAR2 by proteolytic cleavage (57), resulting in activation of intracellular signaling such as MAPK/ERK (54, 58, 59), the latter participating in cell growth, differentiation, and survival (60, 61). When added to resting microglia/optic nerve cocultures, PRSS2 significantly increased the number of TUJ-1 cells. Our microarray data also identified a number of mRNAs that encode secreted proteins that were increased in activated microglia including IL1, CXCL2, and CXCL13. In contrast, SLPI gene expression was significantly reduced in activated microglia. Factors that seemed up-regulated or down-regulated after microarray analyses were tested for their neurogenic effects and, interestingly, the serine protease inhibitor SLPI plays an inhibitory role in neurogenesis, because its addition to cocultures of activated microglia abolished neurogenesis enhancement. Although activated microglia increased expression of cytokines and chemokines related to inflammation and innate immunity, these proteins did not influence neurogenesis in our cell culture model.

Our studies support the concept that activated microglial cells secrete a protein that enhances neurogenesis in neonatal ONCs in vitro. Our studies implicate the serine protease, PRSS2, a precursor of trypsin, in this microglia-mediated neurogenesis. In vivo studies using models of neurodegenerative diseases, microglia activation, and/or trypsinogen as a neurogenic factor may provide direction for the development of therapies that promote neurogenesis in the mammalian brain.

Materials and Methods

Animals. Sprague–Dawley rats (6–8 wk old) were divided into two groups and injected i.p. with 1.0 mg/kg LPS (Sigma) or PBS (control group) once a day for four consecutive days. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Cleveland Clinic.

Microglia Isolation from Rat Brains and Flow Cytometry. Twenty-four hours after the final i.p. injection, rats were anesthetized and transcardially perfused with PBS. Brains were rapidly removed, minced, and trypsinized for 30 min at 37 °C. Trypsin was quenched by adding 20% (vol/vol) FBS in DMEM. The cell suspension was separated on a discontinuous 30/70% percoll gradient. Myelin and cell debris were discarded from the top of the 30% (vol/vol) percoll layer and the cell-enriched fraction at the top of the 70% (vol/vol) percoll was collected. The cells were incubated with fluorescently conjugated CD11b and CD45 antibodies (1:250; BD Pharmingen) at 40 °C for 30 min (Fig. 1*A*). Fluorescently labeled cell populations were separated using a Becton-Dickinson FACSAria I Flow Sorter as described previously (36, 37). Activated microglia were CD11b-positive/CD45-intermediate, whereas resting microglia were CD11b-positive/CD45-low. These microglia populations along with other glial cells (CD11b-negative/CD45-negative) were collected, centrifuged, resuspended in Neurobasal -A media (Gibco), and plated in six-well PLL-coated plates at a density of 2×10^4 cells per well.

Dissociated Cell Culture of Optic Nerve from Rat Pups. Sprague–Dawley rat pups 3 to 6 d old were decapitated and the optic nerves were removed, minced, and placed in 0.1% trypsin and 0.25% EDTA in MEM at 37 °C for 25 min. Enzymatic activity was quenched with 10% (vol/vol) FBS in DMEM and DNase was added to digest nuclear debris. The cells were triturated with a fire-polished Pasteur pipette, filtered through a 40-μm cell strainer, centrifuged at $740 \times g$ for 8 min, and resuspended in 1% FBS/DMEM/Hank's modified N2 (1% vol/vol) and PDGFAA (10 ng/mL; Sigma). Cells were counted using a hemacytometer and were plated on PLL/laminin-coated 12-mm glass coverslips at a density of 3.2×10^4 cells per coverslip. Coverslips were placed in 20-nm-pore-size inserts that in turn were placed in the wells containing the FACS-sorted microglia or other glial cells. Additional media consisting of 1% FBS, 0.1% PDGFAA, and 1% Hank's N2 in DMEM were added in the inserts and the wells containing the microglial cells (Fig. 1*C*).

Immunopanning of A2B5 Cells. Anti-goat IgM-coated Petri dishes were incubated with mouse monoclonal A2B5 antibody diluted 1:1 in 1 mg/mL BSA in DMEM for 2 h at room temperature. Dissociated ONCs prepared as described above were placed in the A2B5-coated Petri dishes for 30 min at 37 °C. The dishes were agitated every 10 min to detach A2B5-negative cells, resulting in a 99% pure OPC population. OPCs were then detached from the Petri dishes, counted with a hemacytometer, and plated on coverslips at a density of 3.2×10^4 cells per coverslip, which were in turn placed in inserts for coculture as described above.

Immunocytochemistry. To phenotype ONCs, double-labeling immunocytochemistry was performed using neuronal (mouse anti–TUJ-1 1:5,000; Covance and rabbit anti-MAP2 1:500 and rabbit anti-GAD65/67 1:250, both from Millipore) and glial markers (rabbit anti-GFAP 1:500; DAKO) in 10% (vol/vol) normal goat serum/0.1% Triton X-100/PBS . For glial cell surface markers (rabbit anti-NG2 1:250; Millipore and mouse anti-A2B5 1:50; Sigma) antibodies were diluted in 10% (vol/vol) NDS/DMEM. Cells were then visualized using an Alexa Fluor IgG_{2A} 594 and a polyclonal Alexa Fluor 488 or Alexa 488 a-mouse IgM (all 1:500; Invitrogen). Details of BrdU immunohistochemistry are given in *SI Materials and Methods*. Coverslips were mounted on slides with Vectashield mounting medium containing DAPI (Vector Laboratories Inc.).

Cell Counting. Ten random fields from each coverslip imaged with a 10× objective were photographed and TUJ-1–positive and DAPI cells were counted. Cell counts were collected using ImagePro software based on threshold intensity. All slides were coded and all subsequent analyses were performed blinded to treatment condition.

Microarray and RT-PCR Procedure. RNA was isolated from FACS-sorted microglial cells using a TRIzol reagent system (Invitrogen) and a Qiagen mRNA kit (Qiagen Inc.). RNA was extracted from five preparations of resting and five preparations of activated microglial cells and probed by genechip analysis using Affymetrix Rat 2.0 genome array according to the manufacturer's instructions (Affymetrix). Microarray data images were analyzed using Genechip operating system software (Affymetrix) and transported into GeneSpring software (Agilent Technologies) to perform univariate and principle component analysis. Microarray probe level normalization of log2-transformed data was performed using robust mean averaging (62) and tested for statistical significance using a two-tailed groupwise *t* test assuming equal variance with Benjamini–Hochberg false discovery rate correction. We selected the initial lots of genes that were 1.5-fold altered across replicates between the two groups of samples. We next used this list of significantly altered genes to query for transcripts related to secreted proteins. Thirty secreted proteins were significantly changed in the comparison between resting and activated microglial cells. The genes are listed in Table S4. For SLPI mRNA analysis, TaqMan multiplex gene expression assays used probes specific for SLPI (Rn00670378_m1; labeled with FAM) and GAPDH (43083130; labeled with VIC) following manufacturer's instructions (Applied Biosystems). All reactions also included no template and negative (no reverse transcriptase) controls. SLPI data were normalized to the respective in-tube GAPDH levels and compared using one-way ANOVA.

Serum Depletion from Microglia Media and Fractionation. Media from resting and activated microglia were collected and concentrated using the 3K Amicon Ultra filtration cellulose (Millipore) by centrifugation at $3,716 \times g$ for 30 min. Concentrated media were then diluted 10 times in TBS (1× dilution buffer; Beckman Coulter) and filtered through 0.45-μm Spin-X filter membranes and added to prepacked ProteomeLab IgY-12 affinity microbead columns (Beckman Coulter) to deplete serum from samples. Columns were then mixed by inversion and placed on an end-to-end rotator for 15 min at room temperature followed by centrifugation to collect the samples. Columns were then stripped of bound proteins by consecutive washes in dilution, stripping, and neutralization buffers (Beckman Coulter).

Mass Spectrometry Proteolysis and Analysis. In-solution proteins were reduced with DTT and alkylated with iodoacetamide prior to the digestion. The samples then were digested with sequencing-grade modified trypsin (Promega), which is specifically modified by reductive methylation, rendering it resistant to proteolytic digestion, at an enzyme-to-protein ratio of 1:20 (wt/wt) in solution at 37 °C for 12 h. The digestion reaction was terminated by adding 1% formic acid. Digested samples were then analyzed with mass spectrometry. Using a column-switching technique, the resulting peptides were loaded onto a 300-μm (i.d.) × 5-mm C18, PepMap RP trapping column to preconcentrate and wash away excess salts for in-solution digested samples. The loading flow was set to 10 μL/min, with 0.1% formic acid used as the loading solvent. Reverse-phase separation was performed on a 75-μm i.d. × 15 cm C18, PepMap column using the nanoseparation HPLC systems (LC Packings and U-3000; Dionex). Buffer A (100% water and 0.1% formic acid) and buffer B [20% (vol/vol) water, 80% (vol/vol) acetonitrile, and 0.1% formic acid] were used to design a gradient. Proteolitic peptides eluted from the column with a gradient of acetonitrile of 2% per minute were directed to a mass spectrometer (LTQ FT; Thermo Finnigan) equipped with a nanospray ion source and with a needle voltage of 2.2 kV. All mass spectra were acquired in the positive ion mode. Dynamic exclusion for 45-s duration time was used to acquire MS/MS data from low-intensity ions.

Database Search. MS/MS data of the peptide mixtures were searched against National Center for Biotechnology Information rat database for the protein ID using the Mascot engine (Matrix Science). Manual examination was carried out for all proteins identified with fewer than three peptides.

In Vitro Protein Treatment. Select proteins identified in the mass spectrometry and microarray studies were added to the media of resting microglia, activated microglia, or culture media without FACS-sorted cells to test whether they would affect neurogenesis in the optic nerve cultures. Proteins were selected based on the genes that showed the highest significant differences on *P* values between resting and activated microglia after the microarray analysis and the mass spectrometry media analysis originating from resting and activated microglia. Proteins used were the following: full-length recombinant human CXCL2 (H00002920-P01; Abnova), full-length recombinant human CXCL13 (H00010563-P01; Abnova), full-length recombinant human trypsin 2 (3586-SE-010; R&D Systems), partial recombinant human secretory leukocyte protease inhibitor (H00006590-Q01; Abnova), and full-length recombinant human interleukin 1β (H00003553-P01; Abnova).

Statistical Analysis. All counted cell numbers were analyzed using single-factor ANOVA (one-way ANOVA) followed by Tukey's post hoc analysis where appropriate. Statistically significant differences were defined as $P < 0.05$.

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