Microglia induce neurogenesis by stimulating PI3K/AKT intracellular signaling in vitro

Kristi Lorenzen
University of Nebraska Medical Center College of Medicine
Nicholas W. Mathy
Creighton University School of Medicine
Erin R. Whiteford
Creighton University School of Medicine
Alex Eischeid
University of Iowa Roy J and Lucille A Carver College of Medicine
Jing Chen
Creighton University School of Medicine
Matthew Behrens
University of Nebraska Medical School
Xian-Ming Chen
Creighton University School of Medicine
Annemarie Shibata (ash33271@creighton.edu)
Creighton University https://orcid.org/0000-0003-2451-3065

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Abstract

Background: Emerging evidence suggests that microglia can support neuronal survival, synapse development, and neurogenesis in classic neurogenic niches. Little is known about the ability of microglia to regulate the cortical environment and stimulate cortical neurogenesis outside classic neurogenic niches. We used an in vitro co-culture model system to investigate the hypothesis that microglia respond to soluble signals from cortical cells, particularly following injury, by altering the cortical environment to promote cortical cell proliferation, differentiation, and survival. Results: Analyses of cell proliferation, apoptosis, protein expression, and intracellular signaling were performed on uninjured and injured cortical cells in co-culture with an EOC2 microglial cell line. Microglia soluble cues enhanced cortical cell viability and proliferation of uninjured and injured cortical cells. Co-culture of injured cortical cells with microglial cells significantly reduced cortical cell apoptosis. Microglial significantly increased Nestin+ and a-internexin+ cells within and outside the injury site. NeuN+ cells increased in injured cortical cultures with microglia. Multiplex ELISA assays showed decreased levels of inflammatory cytokines in conditioned media collected from injured cortical cell and microglial co-culture. RTPCR analysis of microglial mRNA was performed. AKT phosphorylation in uninjured, and particularly injured cortical cells, significantly increased when co-cultured with EOC2 microglia. Inhibition of AKT phosphorylation in cortical cells blocked the microglial-enhanced cortical cell viability and expression of neurogenic markers in vitro . Conclusion: This in vitro model system allows for assessment of the effect of microglial-derived soluble signals on cortical cell viability, proliferation, and stages of differentiation during homeostasis or following injury. These data suggest that EOC2 microglia downregulate inflammatory cytokine production following activation by acute cortical injury to enhance proliferation of new cells capable of neurogenesis. Inhibition of AKT signaling in cortical cells blocks the microglial-derived enhanced proliferation and expression of neurogenic markers in injured cortical cultures. This in vitro system is useful for continued studies with other microglial cell lines and primary microglial cells. Increasing our understanding of the mechanisms that drive cortical neurogenesis stimulated by microglial cells during homeostasis and following injury will provide insight into the potential mechanisms of the neuroprotective role of immune activity in the central nervous system (CNS).

Background

Microglia are resident immunocompetent and phagocytic cells of the central nervous system (CNS) and comprise anywhere from 5-12% of cortical cells [1, 2]. During homeostasis, microglia survey the local CNS environment and communicate with neighboring glia and neurons through membrane bound and soluble signals [3, 4]. Emerging evidence suggests that, given specific activator(s), microglia function to support neuronal proliferation, differentiation, synaptic function, and survival [4,5]. Microglia contribute to synaptic development by refining axonal branching and pruning synaptic connections through phagocytic activity [6-9]. Additionally, specific microglial-derived cytokines, growth factors, and cell associated proteins play an important role in the modification and function of both excitatory and inhibitory synaptic connections in the CNS [10-12].

Microglia support neurogenesis in the classic neural stem cell niches of the subgranular zoneof the dentate gyrus in the hippocampus and the subventricular zone lining the lateral ventricles [13-23]. Further, *in vivo* studies have shown that specifically stimulated microglia, macrophages, and infiltrating T cells protect neuronal axons from secondary degeneration following injury, degrade inhibitory proteins that restrict neuronal survival and

regrowth, reduce pro-inflammatory cytokine production, and induce growth factor and neurotrophin production [24-25]. Production of anti-inflammatory mediators and neurotrophic factors by microglia are likely to be dependent on the nature and duration of the stimulus as well as the severity of injury to which microglia respond [26, 27]. For example, microglia stimulated by damage signals from a human peripheral nerve increase BDNF and GDNF secretion; upregulate the expression of migratory cytoskeletal proteins; upregulate proteolytic and debris clearing enzymes; and, enhance both STAT protein expression and NFκB gene transcription [28]. Microglial proliferation and increased release of transforming growth factor –b (TGF-b) are correlated with neural stem cell proliferation in the adult dentate gyrus [13]. Secretion of insulin growth factor 1 (IGF-1) from microglia following status epilepticus in the adult dentate gyrus stimulates neurogenesis via activation of the p42/44 MAPK pathway [30]. Other work suggests that injury to adult CA1 neurons of the dentate gyrus stimulates IGF-1 release from microglia and astrocytes promoting neuronal survival via AKT phosphorylation and decreased MAPK phosphorylation [31] or via both AKT and MAPK phosphorylation [32]. Increased AKT phosphorylation by PI3K signaling is important for neurogenesis in classic stem cell niches as well as the cortex [31-38]. Recent studies have shown that microglial-derived FGF and EGF growth factor and IL-10/IL-13 cytokine secretion promote the proliferation and differentiation of adult neural stem cells *in vitro* [39]. While an ever-growing body of work supports the role of microglial soluble signals in proper neurogenesis and plasticity, neuroinflammation caused by microglia activity is also linked to neurodevelopmental and neurodegenerative diseases [40-42]. For example, the reduction in neuroinflammatory cytokines such as TNF-α, IFN-Y, MIP-1α and RANTES/CCL5, IL-1α, and IL-1β suppresses apoptosis and enhances neurogenesis [29, 43]. Recent work demonstrates that during normal development and inflammatory states, primary microglia, even in the same region of the brain, express complex patterns of gene expression resulting in functionally diverse microglial phenotypes [43,44]. Taken together these data suggest that a complex milieu of microglial-derived soluble cues with neurogenic or neuroinflammatory properties work in combination to promote or restrict neuronal development, survival, and repair following injury [7, 18, 39-46].

Outside the classic neural stem cell niches, CNS stem cells have the potential to generate neurons and glial cells following ischemia or traumatic injury [35, 47-50]. Microglial-derived soluble cues are potentially important for local endogenous neurogenesis, migration, and neuronal survival [51]. Interestingly, microglial invasion of the cortical plate overlaps with peak periods of cortical neurogenesis [1-3, 52]. After invasion, microglia remain as morphologically and functionally dynamic cells within the environment of the cortex [2-3, 46, 49]. Microglial-derived cytokines may promote neurogenesis by supporting progenitor cell survival and mitosis [53-56]. Given the diverse functional roles that microglia can play in the cortex, the development of an *in vitro* model system to evaluate the neurogenic potential of a uniform population of microglia is important. We hypothesize that microglia stimulate localized neurogenesis of cells throughout the cortex during homeostasis or following injury by activating specific intracellular signaling pathways required for neuronal survival and differentiation. Little is known about the mechanisms by which homeostatic microglia or activated microglia responding to injury influence neurogenesis and survival outside of hippocampal and lateral ventricular neurogenic niches.

In this study we present an *in vitro* model system for investigating the underlying mechanisms by which microglia respond to cortical cues to regulate neuronal differentiation and survival during homeostasis and following acute mechanical injury. Our *in vitro* system utilized an EOC2 microglial cell line suspended above primary rat cortical cells that were injured or left uninjured as control. Primary cortical cells were isolated from Sprague-Dawley rat embryos at embryonic day 16-18 because the differentiation of these cortical cells has been

well characterized *in vitro* [57]. Neurogenesis or the differentiation of new neurons in primary cortical cell culture can be determined by evaluating the progressive expression of neurogenic proteins such as Nestin, GFAP, α-internexin, and NeuN [58,59]. Nestin expression has long been known to be upregulated in neural progenitor cells and cortical radial glia [60]. Several studies also report that Nestin protein is expressed in early differentiating neurons in rodents and humans [61-66]. Recently, Nestin has been shown to be co-expressed with doublecortin in immature neurons of the cortex [67,68]. GFAP expression is associated with early stages of neurogenesis and is co-expressed with Nestin in cells of the neurogenic niche, such as neural stem cells, and following hypoxia in the cortical parenchyma [69]. GFAP is also expressed in populations of mature astrocytes and can be used as a marker for these glial cells. Later in neurogenesis, cytoskeletal elements such as α-internexin and neurofilament are upregulated and highly expressed as neurons begin to mature [70-72]. Alpha-internexin is expressed in postmitotic immature neurons as they commence differentiation and can be found co-localized with neurofilament triplets in mature neurons in the CNS [72]. The neuronal splicing regulator, NeuN, is expressed in both post-mitotic immature and mature cortical neurons [73]. We utilized the well characterized stages of neurogenesis in primary cortical cells to assess the effects of microglia during homeostasis and cortical injury on cortical cell viability, proliferation, differentiation, and intracellular signaling. While this co-culture system investigates the interactions between cortical cells and EOC2 microglial cell lines, the co-culture system can be used with other microglial cells lines and primary microglia.

Our data suggest that EOC2 microglial-derived soluble cues promote cortical cell viability and enhance proliferation of cortical cells. Microglial-derived cues reduced apoptosis of primary cortical cells following acute mechanical injury *in vitro*. In injured cortical cell and microglial co-cultures, significantly increased expression of neurogenic markers Nestin and α-internexin was present within the site of injury where proliferating cells were observed. Expression of the mature neuronal marker NeuN increased in injured cortical cells outside the injury site when co-cultured with microglial cells. AKT phosphorylation was increased in cortical cells co-cultured with EOC2 microglial cells. Inhibition of AKT phosphorylation reduced the enhanced expression of neurogenic markers in cortical cell and microglial co-cultures. EOC2 microglial cells responding to acute injury downregulated their expression of pro-inflammatory cytokines. These results show that this co-culture *in vitro* system provides a model to evaluate cortical cell responses to microglial derived soluble cues and to investigate the underlying mechanisms of the functional states of microglia in response to cortical signals during homeostasis or following injury. EOC2 cell lines provided an example of how microglial cells may influence cortical cell differentiation following injury outside of neurogenic niches. This co-culture system provides a useful tool to further investigate the neuroimmune mechanisms important for primary microglial responses and cortical cell differentiation and survival *in vivo*.

Results

The *in vitro* system utilized EOC2 microglial cells cultured on Transwell® inserts suspended directly above uninjured or injured primary cortical cells. These co-cultures were established to investigate the effect of microglial responses on cortical cell proliferation, survival, and differentiation during states of cortical cell homeostasis and following mechanical injury. Primary cortical cultures were established using methods previously described [57]. To characterize the cortical cell types at the time of microglial co-culture, immunocytochemical analysis of cortical cell protein expression was performed at two days *in vitro* (2DIV, Figure 1). In the control cortical culture, 56.3 ± 0.3% of cells expressed Nestin, 51.3 ± 2.0% expressed α-

internexin, 41.7 ± 0.3% of cells expressed the mature neuronal marker TUJI, and 4.3 ± 0.3% expressed glial fibrillary acidic protein (Figure 1D). Only 1.9 ± 0.6% of over 1000 cells counted were immunopositive for the microglial marker CD11b (CD11b+) demonstrating that the culture conditions did not support primary microglial cell proliferation and survival (Figure 1 E; over 300 cells were counted per experiment in three separate experiments; ± represents standard error of the mean (SEM)).

Immediately prior to co-culture with EOC2 microglial cells, cortical cells were injured using a sterile stylet to disrupt and remove cortical cells from the cell culture surface [74]. Uninjured cortical cells were used as controls. Uninjured and injured cortical cells were then cultured for two additional days with or without EOC2 microglial cells on Transwell® inserts. The site of injury (indicated by the dashed white line) was observable and few neurofilament immunopositive (NF+) cells or processes were found in the injury site in injured cortical cultures without microglia (Figure 2A, B; black scale bar represents 100 μm). In cortical cultures with EOC2 microglia, the site of injury (indicated by the dashed white line) was associated with increased cell density and increased neurofilament expression at the site of injury (Figure 2C, D). EOC2 microglial cells used for co-culture experiments are CD11b+ (Figure 2 E, F; white scale bar represents 50 μm).

Cortical cell viability following injury and co-culture with microglia was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) colorimetric assays that measure metabolic activity in living cells. Injured cortical cultures without EOC2 microglial cells and uninjured cortical cultures with and without EOC2 microglial cells were also assessed using the MTT assay. Quantification of optical density (O.D.) of three separate MTT assays performed in triplicate shows that in the absence of cortical cell injury, microglial-derived soluble cues significantly enhanced cortical cell mitochondrial activity by a mean difference of 0.28 ± 0.03 O.D. units (*$p<0.05$, ± represents SEM, n=3) as compared to uninjured cortical cells cultured in control media alone (Figure 2G). Co-culture of injured cortical cells with EOC2 microglial cells significantly increased mitochondrial activity by a mean difference of 0.39 ± 0.03 O.D. units (*$p<0.05$, ± represents SEM, n=3) when compared to uninjured conditions without EOC2 microglial cells and by 0.42 ± 0.02 O.D. units (**$p<0.01$, ± represents SEM, n=3) when compared to injured cortical cells co-cultured without EOC2 microglial cells (Figure 2G). The metabolic activity of injured cortical cells alone was not significantly different from MTT activity in uninjured cortical cell control conditions (Figure 2G, $p>0.05$, n=3).

Immunocytochemical assays specific for measuring cell proliferation and survival were used to examine the response of primary cortical cells to co-culture with soluble signals from EOC2 microglial cells. Cell proliferation was measured by incorporation of a modified, fluorescently labeled thymidine analogue EdU into newly synthesized DNA. Large field confocal image analysis of uninjured cortical cells without EOC2 microglial cells showed the presence of EdU+ cells demonstrating that these cultures had at least a limited number of dividing cells upon isolation from the cortex (Figure 3A). In the presence of EOC2 microglial cells, the number of EdU+ cells increased in uninjured cortical cell culture (Figure 3B). Mechanical injury of cortical cells stripped away cortical cells from the culture surface as indicated by dashed white lines (Figure 3C-D). Without EOC2 microglial cells, few EdU+ cells were observed in the damaged area (Figure 3C). When injured cortical cells were co-cultured with EOC2 microglial cells, an increase in proliferating EdU+ cells was seen throughout the culture and within the damaged area (Figure 3D). Full magnification of the boxed area within the injured site and EdU+ cells (Figure 3D) is shown in Figure 3E. Quantification of the percent of proliferating cells in uninjured cortical culture without EOC2 microglial cells showed that 45.7 ± 5.0% of the cells were EdU+ (Figure 3F). In the presence of EOC2 microglial cells, the average percent of EdU+ cells increased to 74.3 ± 5.6%. This 28.6 ± 7.5% increase in

EdU+ cells in the presence of EOC2 microglial cells was significant (Figure 3F, *p< 0.05, ± represents SEM, n=3). Following injury, the percent of EdU+ cells in cortical cultures without EOC2 microglial cells was 47.2 ± 9.3% and was not significantly different from the control, uninjured cortical cells cultured without EOC2 microglial cells (Figure 3F, p>0.05, ± represents SEM, n=3). When cultured with EOC2 microglial cells, the percent of proliferating EdU+ cells in injured cortical cultures was 84.3 ± 3.3%. This increase was significantly different from the percent of EdU+ cells in uninjured control conditions (Figure 3F, **p<0.01, ± represents SEM, n=3) and from the percent of EdU+ cells in injured conditions without microglia (Figure 3F, *p<0.05, ± represents SEM, n=3) . The difference in the percent of proliferating cells between uninjured and injured cortical cells co-cultured with microglia did not reach significance (Figure 3F, p>0.05, n=3).

To evaluate the effect of EOC2 microglial cells on cell survival, Click-iT® fluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed. TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. Figure 4 illustrates TUNEL+ immunocytochemistry observed using large field confocal imaging of uninjured and injured cortical cultures in the presence or absence of EOC2 microglial cells. In the absence of EOC2 microglial cells, very few TUNEL+ cells were present in uninjured cortical cell cultures (Figure 4A). Following injury, cortical cells in the absence of EOC2 microglial cells, showed increased TUNEL expression particularly in the area of damage (Figure 4C). When injured cortical cells were co-cultured with EOC2 microglial cells, a decrease in TUNEL+ cells were observed both within the injured area and throughout the cell culture (Figure 4D). Full magnification of the boxed area within the injury site clearly revealed the presence of TUNEL+ cells (Figure 4E). Quantification of TUNEL staining in uninjured cortical cultures without EOC2 microglial cells shows that 1.72 ± 0.2% of the cells were TUNEL+. Co-culture of EOC2 microglial cells with uninjured cortical cells did not significantly alter the percent of TUNEL+ cells (3.44 ± 0.6%, ± represents SEM, n=3, p>0.05, Figure 4F). Following injury, the percent of TUNEL+ cells in cortical cultures without EOC2 microglial cells significantly increased to 30.1 ± 4.9% (± represents SEM, n=3, *p<0.05, Figure 4F). When cultured with EOC2 microglial cells, the number of TUNEL+ cells in injured cortical cultures decreased to 5.6 ± 1.2% (± represents SEM, n=3, Figure 4F). The reduction of TUNEL staining by 24.6 ± 4.8% in injured cortical cells co-cultured with microglia was highly significant (**p<0.01, ± represents SEM, n=3, Figure 4F). The percent of TUNEL+ cells in uninjured cortical cultures in the presence of EOC2 microglial cells was not significantly different from the percent of TUNEL+ cells observed in uninjured cortical cultures used as control (p>0.05, n=3, Figure 4F).

Since EdU expression was increased and TUNEL expression was reduced in injured cortical cells co-cultured with EOC2 microglial cells, we sought to determine whether the proliferating cells were expressing neurogenic protein markers. Progressive expression of proteins such as Nestin, glial fibrillary acidic protein (GFAP), α-internexin, and NeuN is indicative of stages of neurogenesis [58-73]. Quantification of Nestin+, glial fibrillary acidic protein (GFAP)+, α-internexin+, and NeuN+ cortical cells following injury and exposure to EOC2 microglial cells was determined using immunocytochemical and western blot analyses.

In order to address whether microglial derived soluble signaling cues influence the number of cells expressing Nestin, GFAP, α-internexin, and NeuN, co-culture experiments were established where cortical cells were injured or left uninjured and then cultured with EOC2 microglia as previously described. After 2DIV, cortical co-cultures were fixed and immunostained for Nestin, GFAP, α-internexin, and NeuN. Nuclei were identified with DAPI (blue immunofluorescence). In all experiments at least 100 cells were counted per field from three separate culture fields for each condition. The percent of immunopositive cells per field was determined by dividing the number

of immunopositive cells by the total number of DAPI stained cells per field. The average percent of immunopositive cells was determined for three biological replicates and compared to controls. Two-way ANOVA with multiple comparisons followed by Tukey's multiple comparisons test was used to determine significance of these data. The presence of EOC2 microglial soluble cues significantly increased the percent of Nestin+ cells in uninjured cortical cultures by ~23% (Figure 5A, ***$p<0.001$) as compared to control, uninjured cortical cultures without microglia. Microglial soluble cues increased the percent of Nestin+ cells within the site of injury by more than 45% and by approximately 38% outside the site of injury as compared to control, cortical cultures without EOC2 microglia. These differences were highly significant (Figure 5A,****$p<0.0001$). Injury of confluent cortical cultures without EOC2 microglial soluble cues resulted in a significant loss of more than 20% of Nestin+ cells within the injury site while no significant change in the percent of Nestin+ cells outside the injury site was observed (Figure 5A). Following injury, co-culture with EOC2 microglial soluble cues significantly increased the percent of Nestin+ cells at the site of injury by over 22% and outside the site of injury by approximately 15% (Figure 5A, ***$p<0.001$ and *$p<0.05$). In all culture conditions, the percent of GFAP+ cells that increased in the uninjured and injured cortical cultures with EOC2 microglia was not significantly different from that of control, uninjured cortical cells without microglial co-culture (Figure 5B, $p>0.05$). The expression of α-internexin, an intermediate filament expressed in post-mitotic and mature neurons, was significantly increased in cortical cultures when in the presence of EOC2 microglial soluble cues (Figure 5C). The percent of α-internexin+ cells in uninjured cortical cells increased nearly 17% in the presence of microglial-derived soluble cues as compared to controls (Figure 5C, *$p<0.05$). Injury of cortical cultures in the absence of EOC2 microglia decreased the percent of α-internexin+ cells by nearly 32% (Figure 5C, ****$p<0.0001$). Co-culture of injured cortical cells with microglial-derived soluble cues significantly increased the percent of α-internexin+ cells by nearly 73% within the site of injury to 88.8 ± 4.3% and over 74% outside the site of injury to 89.8 ± 4.1% (Figure 5C, ***$p<0.001$ and ****$p<0.0001$, respectively). The percent of NeuN+ cells in uninjured cortical cultures in the presence of EOC2 microglia increased by approximately 5% as compared to uninjured cortical cultures without microglia (Figure 5D, *$p>0.05$). Injury of cortical cells significantly decreased the percent of NeuN+ cells by nearly 20% at the site of injury as compared to uninjured controls in the absence or presence of microglia (Figure 5D, ***$p<0.001$ and **$p<0.01$, respectively). Outside the site of injury, the percent of NeuN+ cells in cultures without microglia was not significantly different from uninjured control conditions (Figure 5D, *$p<0.05$) and was approximately 20% higher than the percent of NeuN+ cells seen within the injury site (Figure 5D, ***$p<0.001$). The presence of EOC2 microglia increased the percent of NeuN+ cells outside the site of injury by approximately 19% to 48.5 ± 1.1% as compared to sites outside of injury without EOC2 microglial in co-culture (Figure 5D, **$p<0.01$). The increase in NeuN+ cells in the presence of microglia following injury is of interest since expression of neurogenic markers should lead to neuronal differentiation as indicated by NeuN expression. Higher power images of NeuN immunoreactivity show NeuN+ cells at the site of injury and outside the side of injury (Figure 1S). An increase in the number of total cells was seen in cortical cultures in the presence of EOC2 microglial-derived soluble cues as compared to those cortical cultures alone (Figure 1SA, B). Several cells within the injury site show low NeuN immunoreactivity and did not meet the criteria for positive immunoreactivity (Figure 1SB).

Analysis of the relative immunofluorescence units (RFU) of immunocytochemical experiments was performed to assess the level of neurogenic protein expression. RFU for each labeled primary and secondary antibody conjugate directed against a neurogenic protein was calculated by measuring pixel intensity for each fluorochrome in images acquired with the same exposure settings for all experimental conditions. For each protein, the immunofluorescence of at least 100 cells per field from three separate culture fields was averaged

and compared to controls. These experiments were performed using cortical cultures from three biological replicates (Figure 6). Following injury, cortical cells were cultured in the presence of EOC2 microglial cells or in media alone. Immunofluorescent images for Nestin and GFAP, α-internexin and GFAP, or NeuN and GFAP showed increased expression of early neurogenic proteins in injured areas when co-cultured with EOC2 microglial cells as compared to media alone. Nestin and GFAP expression was significantly enhanced in injured neuronal cultures co-cultured with EOC2 microglial cells as compared to cortical cells cultured alone (Figures 6A). Cells immunopositive for both Nestin (green) and GFAP (red) are indicated by yellow immunofluorescence. Nestin immunofluorescence increased 7.4 ± 0.3fold in injured areas of cortical cells co-cultured with EOC2 microglia as compared to Nestin immunofluorescence in injured cortical cultures without EOC2 microglial co-culture (± represents SEM, n=3, ****p<0.0001, Figure 6B). A 4.0 ± 0.2fold increase in GFAP immunofluorescence was observed in injured cortical cultures co-cultured with EOC2 microglial cells as compared to controls (± represents SEM, n=3 ***p<0.001, Figure 6B). Expression of α-internexin, significantly increased 16.7 ± 0.8fold in injured cortical cultures when exposed to microglial-derived soluble cues as compared to injured controls alone (± represents SEM, n=3, ****p <0.0001, Figure 6B). NeuN immunofluorescence was 0.4 ± 0.1fold higher in injured cortical cultures with EOC2 microglial cells than in control cultures without microglia at 2 DIV (± represents SEM, n=3 *p<0.05 Figure 6B).

Western blot analysis was used to assess protein expression of neurogenic markers in injured as well as uninjured cortical cultures (Figure 7A). Nestin protein expression in uninjured and injured cortical cultures increased 1.7 ± 0.1fold and 1.5 ± 0.1fold respectively following co-culture with EOC2 microglial cells as compared to expression in uninjured control cultures alone (± represents SEM, n=3, ****p<0.0001, ***p <0.001, Figure 7B). No significant change in Nestin expression was observed in cortical cultures that were injured and not co-cultured with EOC2 microglial cells (p>0.05, n=3, Figure 7B). A 1.9 ± 0.5fold increase in α-internexin expression was observed in uninjured cortical cultures co-cultured with EOC2 microglial cells as compared to control, uninjured neurons cultured alone (± represents SEM, n=3, **p <0.01, Figure 7B). Injured cortical cells co-cultured with EOC2 microglial cells exhibited a 2.4 ± 0.4fold increase in α-internexin expression compared to control, uninjured neurons cultured without EOC2 microglial cells (± represents SEM, n=3**p <0.01, Figure 7B). No significant change in Nestin expression was observed in cortical cultures that were injured and not co-cultured with EOC2 microglial cells (p>0.05, n=3, Figure 7B). GFAP, expression increased 1.6 ± 0.1fold in uninjured cortical cells co-cultured with EOC2 microglial cells and 1.9 ± 0.2fold in injured cortical cells co-cultured with EOC2 microglial cells as compared to control uninjured cortical cells (± represents SEM, n=3, ***p <0.001, Figure 7B). Expression of GFAP also significantly increased ~1.6 fold in injured cortical cells without microglial co-culture (***p<0.001, n=3, Figure 7B). Injured cortical cells alone showed a 0.25 ± 0.1fold decrease in NeuN expression as compared to uninjured control cortical cultures (± represents SEM, n=3, *p <0.05, Figure 6B). NeuN protein expression in injured cortical cells co-cultured with EOC2 microglial cells was not significantly different than NeuN expression in control conditions as determined by western blot analysis of total protein (± represents SEM, n=3, p>0.05, Figure 7B).

In order to begin to examine changes in the cytokine environment of cortical cells in the presence of EOC2 microglia, multiplex ELISA assays were used to determine the presence of well-characterized cytokines in microglial-conditioned media. Media collected from co-cultures of injured cortical cells and EOC2 microglia was compared to media collected from co-cultures of uninjured cortical cells and EOC2 microglia. Analysis of three separate assays performed in triplicate showed that the concentration of several cytokines was significantly

different from the levels observed in EOC2 microglial cells-conditioned media when suspended above uninjured cortical cells (Figure 8). The concentration of cytokines measured in media collected from uninjured cortical cell and microglial co-culture was used as the baseline, normalized, and set equal to one (Figure 7A). When compared to control cytokine concentrations, MCP-1 concentration increased 22.0 ± 0.02% above control levels while IFN-g and TNF-a expression concentration decreased to 41.3 ± 0.07% and 73.5 ± 0.08% below control levels, respectively (± represents SEM, n=3, *p <0.05, Figure 8A). Concentrations of MIP-1a and RANTES decreased by ~20% in media from injured cortical and microglial co-cultures compared to media from uninjured cortical and microglial co-cultures but these decreases were not significant (p>0.05, Figure 8B). IL-1a, IL-1b, IL-2, IL-4, IL-6, and GM-CSF were either undetectable or not significantly different in conditioned media from uninjured and injured cortical and microglial co-cultures. IL-1a, IL-1b, IL-2, IL-4, IL-6, and GM-CSF were either undetectable or not significantly different in conditioned media from uninjured and injured cortical and microglial co-cultures (Figure 2S). IL-1a, IL-1b, IL-2, IL-4 levels were undetectable in conditioned media from uninjured and injured cortical cultures with and without microglia. IL-6 was measured to be 3.5 ± 14.4 pg/ml (± is SD) in uninjured cortical cell and microglia conditioned media. The large standard deviation does not allow for accurate interpretation of IL-6 in this co-culture system. GMCSF was also detected in uninjured and injured cortical cell and microglia co-culture media. GMCSF concentration was 18.2 ± 13.2 pg/ml (± is SD) and 14.1 ± 16.3 pg/ml (± is SD) in uninjured cortical cell and injured cortical microglial co-culture media respectively (Figure 2S). The large standard deviation does not allow for accurate interpretation of GMCSF in this co-culture system (Figure 2S).

To begin to investigate whether the significant differences in cytokine concentrations detected in our microglial-conditioned medias are due to changes in microglial cytokine expression, RT-PCR was used to compare mRNA levels of EOC2 microglial cells following co-culture with injured or uninjured cortical cells. Since EOC2 microglial cells are physically separated from cortical cells by TranswellÒ permeable supports, RNA can be specifically isolated from EOC2 microglial cells upon removal of the TranswellÒ from the cortical culture. EOC2 microglial cells in co-culture with injured cortical neurons demonstrated decreased mRNA expression as compared to controls (indicated by the dashed line) for IFN-g (decreased by 22.2±10.2%), MCP-1 (decreased by 79.7 ± 2.9%), MIP-1α (decreased by 60.2 ± 6.7%) TNF-a (decreased by 97.6 ± 4.1%) and RANTES (decreased by 62.5 ±11.6%) (± is SEM, Figure 8B). Decreased expression of MCP-1 mRNA in EOC2 microglial cells suggests that the increase in MCP-1 protein levels was not microglial derived (Figure 8B). Given that microglial cells, neurons, and astroglial cells can secrete a variety of soluble factors, studies directed at identifying neurogenic microglial, neuronal, or glial soluble signals are being addressed using this *in vitro* system.

Several signaling pathways are activated by soluble signaling molecules during neurogenesis [29, 57-58] and may also underlie the microglial-enhanced neurogenesis observed in our co-culture system. To begin to investigate possible signaling pathways important for microglial-enhanced neurogenesis, injured and uninjured cortical cells in co-culture with and without EOC2 microglial cells were treated with inhibitors for intracellular signaling pathways. We then used the MTT viability assay to screen for those inhibitors that blocked microglial-enhanced viability of cortical cells. Microglial co-culture increased viability of uninjured and injured cortical cultures as compared to cortical cultures alone as shown previously (***p <0.001, see supplemental data, Figure 3SA-D). Inhibitors for MEK (PD98059), p38 MAPK (SKF86002), PKCα/βI/βII/γ (GF109203X), and Janus Kinase 2 protein (AG490) did not block the increased metabolic activity and viability of cortical cells co-cultured with EOC2 microglial cells. AG490 and GF109203X at 40 μM did significantly influence viability of cortical cultures

but these affects were not specific for microglial-enhanced viability (Figure 3S). LY294002, an inhibitor of PI3K, and downstream AKT phosphorylation, specifically reduced microglial-enhanced cortical cell viability by ~50% or 0.52 ± 0.02 and 0.51 ± 0.02 optical density (O.D.) units at 10 and 40μM respectively as compared to untreated, uninjured cortical cells co-cultured with EOC2 microglial cells (± represents SEM, n=3, ****p <0.0001, Figure 9A). Following injury, 10 and 40μM LY294002 treatment significantly reduced microglial-enhanced cortical cell viability by ~73% or 0.73 ± 0.02 and 0.74 ± 0.02 O.D. units (± represents SEM, n=3, ****p <0.0001, Figure 9A). Treatment of uninjured or injured cortical cells in the absence of EOC2 microglial cells with LY294002 did not significantly affect metabolic activity as measured by MTT (± represents SEM, n=3, p >0.05, Figure 9A). Western blot analysis of cortical cells confirmed that the presence of EOC2 microglial cells in suspension above cortical cultures increased phosphorylation of AKT, a PI3K target, in cortical cells (Figure 9B). Analysis of western blots showed that the presence of EOC2 microglial cells in uninjured cortical cultures increased AKT phosphorylation 3.6 ± 1.0fold as compared to uninjured cortical cells alone (± represents SEM, n=3, *p <0.05, Figure 9C). Injury alone did not significantly increase (~0.8fold) phosphorylation of AKT as compared to control levels (Figure 9C, p>0.05). Following injury and co-culture with EOC2 microglial cells, AKT phosphorylation increased 5.0 ± 1.0fold as compared to injured cortical cells alone (± represents SEM, n=3, **p <0.01, Figure 9C). This increase was also significantly different from AKT phosphorylation levels measured in injured cortical cells without microglial co-culture (4.2 ± 1.0fold increase, *p<0.05, ± represents SEM, n=3, Figure 9C). These experiments suggest that phosphorylation of AKT may be necessary for microglial-enhanced expression of specific neurogenic proteins.

To further investigate the necessity of the AKT phosphorylation for microglial-enhanced expression of neurogenic proteins, immunocytochemical analysis of neurogenic protein expression was assessed in injured and uninjured cortical cells co-cultured with EOC2 microglial cells in the presence of 40mM LY294002. Uninjured cortical cells incubated with LY294002 but without EOC2 microglial cells served as the control and baseline for the normalization of protein expression. Incubation of cortical cultures with LY294002 completely blocked the increase in AKT phosphorylation and reduced neurogenic protein expression seen in cortical cells when cultured with EOC2 microglial cells (Figure 9D). LY294002 reduced Nestin expression by 0.44 ± 0.3fold in uninjured cortical cells co-cultured with EOC2 microglial cells as compared to controls (**p<0.01, n=3, Figure 9E). LY294004 treatment reduced Nestin expression by 0.26 ± 0.0fold in injured cortical cells co-cultured with EOC2 microglial cells as compared to uninjured controls (***p<0.001, n=3, Figure 9E) and by 0.36 ± 0.1fold in injured cortical cells cultured without EOC2 microglial cells (**p<0.01, n=3, Figure 9E). Expression of the neuronal intermediate filament α-internexin was significantly reduced by 0.59 ± 0.1fold (****p<0.0001) in injured cortical cells co-cultured with EOC2 microglial cells when compared with uninjured controls and by 0.40 ± 0.1fold (**p<0.01) when compared with injured cortical cells not cultured with EOC2 microglial cells (Figure 9E, n=3). Additionally, microglial-enhanced expression of GFAP in uninjured and injured cortical cells was significantly reduced following treatment with LY294002 (Figure 9D and 9E). GFAP expression was reduced by 0.76 ± 0.0fold as compared to uninjured controls and 0.85 ± 0.1fold as compared to injured cortical cells without microglial co-culture (****p<0.0001, n=3, Figure 9E).

Immunocytochemical analysis was also used to evaluate the effect of blocking PI3K activity and AKT phosphorylation (Figure 10). Co-cultures were established as previously described and cortical cells were incubated with LY294002. After co-culture, cells were fixed and evaluated for the expression of the neurogenic markers- Nestin, a-internexin, and GFAP (Figure 10A) since the expression of these markers was enhanced in our

co-culture system. DAPI immunofluorescence was used to identify nuclei (Figure 10A). Immunocytochemical analysis supported western blot data showing that LY294002 treatment and inhibition of AKT phosphorylation reduced Nestin, α-internexin, and GFAP expression in injured cortical cells co-cultured with EOC2 microglial cells (Figure 10A). Quantification of neurogenic protein expression was determined by calculating RFU for each labeled primary and secondary antibody conjugate directed against neurogenic proteins in images acquired with the same exposure settings for all experimental conditions as described previously. Evaluation of RFU for Nestin expression showed that co-culture with EOC2 microglial cells significantly enhanced Nestin in uninjured or injured cortical cultures as compared to Nestin expression in cortical cultures alone (*$p<0.05$, Figure 10B). Application of 40 mM LY294002 reduced Nestin expression in co-cultures of EOC2 microglial cells and uninjured cortical cells by 8.5 ± 2.6 RFU or ~44% (***$p<0.001$, ± is SEM, n=3, Figure 10B). In co-cultures of EOC2 microglial cells and injured cortical cells, 40mM LY294002 reduced Nestin expression by 17.3 ± 3.9 RFU or ~77% (***$p<0.001$, ± is SEM, n=3, Figure 10B). LY294002 application did not significantly affect Nestin expression in cortical cells that were not cultured with EOC2 microglial cells ($p>0.05$, n=3, Figure 10B). Expression of the neurofilament a-internexin was similarly reduced by inhibiting the PI3K pathway by 12.0 ± 1.9 RFU or ~40 % (***$p<0.001$, n=3) in injured neuronal co-cultures with EOC2 microglial cells (Figure 10C). GFAP expression was also significantly reduced to 26.4% of control. LY294002 did not significantly change protein expression in uninjured or injured neurons cultured in control media alone ($p>0.05$, Figure 10D). These data underscore the importance and necessity of AKT phosphorylation via PI3K activity for microglial-enhanced neurogenesis in this *in vitro* system.

Discussion

This study examines the ability of EOC2 microglial cells to support the viability, proliferation, neurogenesis, and survival of primary cortical cells outside of the classic neurogenic niche during homeostasis and following mechanical injury. Stem cell progenitors outside of the classic neurogenic niches have the capacity for neurogenesis during normal homeostasis and following injury or disease given exposure to the proper combination of neurogenic cues [47, 48-50]. Cortical microglial cells are a potential source of neurogenic signaling molecules [4, 39-42, 51-56]. Microglia have been shown to contribute to neuronal synapse development, survival, and neurogenesis in neurogenic niches of the CNS during development and following injury [5-6, 8, 10-23, 51-56]. Neurogenic potential of microglial-derived cues may be controlled by the mechanism and duration of activation since infection, trauma, and disease also stimulate pro-inflammatory responses from microglia that can lead to neurotoxicity and neurodegeneration [26-27, 43, 75,76]. Several *in vitro* studies suggest that microglial-conditioned media is neurotoxic depending on the mechanism of microglial activation [43, 44-46, 75, 76]. Recent characterization of primary microglia using single cell sequencing and other methods shows that microglia are highly diverse and that subpopulations of functional microglia are present within specific brain regions and in responses to particular environmental cues [45,46]. Because of the diversity of primary microglia, this study presents an *in vitro* model system using the EOC2 microglial cell line and primary cortical cells in co-culture to begin to examine the neurogenic potential of microglial soluble cues during cortical cell homeostasis and following cortical cell injury. Suspension of microglia on Transwell® cell culture inserts directly above cortical cells allows for evaluation of how the cortical environment is influenced by microglial-derived soluble cues. The methods of isolating primary cortical cells utilized in this *in vitro* system have been well characterized [52] and our data show that these cells express primarily neurogenic and neuronal cell markers with evidence of less than 5% astrocytic protein expression and less than 2% microglial protein

expression (Figure 1F). Using this system where EOC2 microglia and primary cortical cells are not in direct contact also allows for protein and RNA to be collected independently from either cell population. This reductionist *in vitro* system allows for preliminary questions to be asked about microglial neurogenic potential in the cortex and can be used for future studies with other microglial cell lines and primary microglial cells.

Results from experiments presented here show that soluble cues from EOC2 microglial cells responding to primary cortical cells enhanced proliferation, suppressed apoptosis, and promoted the expression of neurogenic and mature neuronal markers in the primary cortical cultures. Immunocytochemical analysis and MTT assays of cortical cells in co-culture with suspended EOC2 microglial cells following injury showed that EOC2 microglial cells enhanced viability of Neurofilament+ cells and increased extension of Neurofilament+ processes into injured areas of cortical cultures (Figure 2). Neurofilament is expressed in neuronal progenitor cells and mature neurons and is associated with structural maturation of neurons and axonal function [69, 71] supporting these data showing that microglial-derived cues can promote neurogenic protein expression in cortical cells outside of neurogenic niches.

To more specifically address how EOC2 microglial cells influence cortical cell differentiation, the *in vitro* system was used to assess cell proliferation, apoptosis, and expression of neurogenic markers in uninjured and injured co-culture conditions. Immunocytochemical analyses revealed that co-culturing uninjured or injured cortical cells with EOC2 microglial cells significantly increased the percent of EdU+ cells in cortical cultures as compared to controls (Figure 3). EdU+ cells were observed within the injury site and throughout the culture (Figure 3). Particularly striking is the proliferation of cortical cells within the injury site. Enhanced proliferation is not seen within the injury site in injured cortical cultures without microglia (Figure 3). Microglial responses in the subgranular and subventricular zones can influence the number of mature neurons that are generated by regulating neuronal stem cell and neuronal progenitor cell proliferation and differentiation throughout life [4-5, 10-11, 21, 39, 44]. In the subgranular zone and subventricular zones the microglial responses are dependent upon location and specific developmental time points [4-5, 51-52]. Enhancement of cortical cell proliferation outside of the neurogenic niche as shown using this *in vitro* system suggests that the microglial-derived soluble environment of cortical cells is able to significantly support cortical cell proliferation during homeostasis and following injury (Figure 3).

Apoptosis as measured by TUNEL staining showed that co-culture with EOC2 microglial cells significantly reduced apoptosis in cortical cells following injury (Figure 4). Microglia can influence apoptosis in neurogenic zones and this response is dependent upon the combination of proinflammatory cytokines, growth factors, and phagocytic activity of microglia [5, 22-23, 26-27, 29]. Recently it has been shown that neuroblasts can be recruited from the subventricular zone of hippocampus to sites of injury in the cortex by microglial-derived specific cues suggesting that microglia, at least at specific times following injury, function to attract differentiating neuronal progenitors into cortical tissue [77]. Further studies suggest microglia may also act locally to stimulate neurogenesis of responsive cortical cells at noncanonical neurogenic regions [47, 51-56, 79]. We propose that this *in vitro* model system is useful to investigate whether microglial soluble signals following cortical injury have neurogenic potential outside of previously investigated subventricular and subgranular zones. Further this *in vitro* system allows for analysis of neurogenic protein expression and activation of intracellular signaling pathways mediated by microglia in cortical cells.

EOC2 microglial-derived soluble cues influence neurogenic marker protein expression in cortical cells *in vitro*. The percent of cortical cells expressing Nestin, a-internexin, GFAP, and NeuN and the relative amount of Nestin, a-internexin, GFAP, and NeuN protein expression were significantly higher in uninjured and injured cortical cultures with EOC2 microglia as compared to control cultures without microglia. Nestin expression alone or Nestin and GFAP expression are characteristic of early, primary neurogenic stem cells and progenitors in classic neurogenic regions [2,60-63, 80-81].

Nestin+ progenitors can eventually give rise to intermediate progenitors that produce immature neurons or neuron-committed progenitors [60-68, 80-81]. Nestin and GFAP are also co-expressed by migratory astrocyte progenitors in the neurogenic niche of the subependymal zone following trauma [55-56, 82-83]. Microglial-derived soluble signals may help shape a local environment that can stimulate Nestin+ and GFAP+ cell proliferation important for generation of new neurons and glia following injury in the cortex outside of classic neurogenic regions. The expression of a-internexin, a Type IV neuronal intermediate filament protein, occurs during later stages of neuronal differentiation and axon development [69-71]. Immunocytochemical and western blot results showed that a-internexin expression was significantly increased in injured neurons following co-culture with EOC2 microglial cells. The increase in a-internexin+ cells and expression at the injury site suggests that microglial responses to injury creates an environment that supports early stages of neurogenesis (Figure 5-7). This effect was not limited to the injury site since an increase in the percentage of a-internexin+ cells was also seen outside of the injury site. Neuronal progenitor cells expressing a-internexin were clearly distinct from GFAP expressing cells. Immunocytochemical analysis of GFAP immunoreactivity in injured cortical cultures co-cultured with EOC2 microglial cells showed an increase in GFAP+ cells with the morphology of mature astrocytes as compared to injured cortical neurons alone (Figure 5-6). Western blot analysis confirmed a significant increase in GFAP protein expression in injured cortical cells cultured with EOC2 microglial cells (Figure 7). It is possible that microglia responding to injury stimulate neurogenesis and astrogliogenesis. Microglial soluble signals have been shown to stimulate astrocyte differentiation from progenitor cells [55-56].

In order to determine whether EOC2 microglial cells were able to promote differentiation of mature neurons and enhance the survival of existing neurons following injury we used immunofluorescent and western blot analysis to visualize NeuN expression. NeuN is a marker for mature neurons [73]. The percent of NeuN+ cells increased in injured cortical cell and microglial co-cultures (Figure 5D). Enhanced NeuN immunofluorescence was observed in injured cortical cultures exposed to microglial-conditioned media (Figures 6, 1S). Few NeuN+ cells are present in injured areas when neurons are cultured in control media. Western blot analysis showed an increase in NeuN expression when uninjured or injured neurons were exposed to microglial-conditioned media but this increase did not reach significance when compared with NeuN expression in cortical cultures without EOC2 microglial cells (Figure 7A-B). There are several possible explanations for this result. For western blot analysis, protein was isolated from the entire neuronal culture which included injured as well as uninjured regions and would be less specific for detecting proliferation and maturation in injured areas alone. Maturation of neuronal progenitors takes up to one week in culture [57-58]. The outcome of increased Nestin, GFAP, and a-internexin will require longer culture conditions. Our supplemental data showing increased NeuN immunofluorescence in the area of injury following cortical co-culture with EOC2 microglial cells, suggests that microglial-enhanced neurogenesis and differentiation of mature neurons is occurring (Figure 1S, 2S). Additional, long-term experiments are underway to determine whether maturation of mature neurons results from effector microglial-enhanced neurogenesis. Taken together, these data suggest that microglial soluble

signals released following co-culture with cortical cells during homeostasis and more so during activation by cortical injury promote the proliferation and survival of neurogenic cells.

Multiplex ELISA assays of microglial-conditioned media from co-culture experiments with uninjured and injured cortical cells revealed that the expression of several cytokines significantly changed following EOC2 microglia stimulation by neuronal injury. Specifically, multiplex ELISA data showed significant upregulation of MCP-1/CCL2 and downregulation of IFN-g, MIP-1a, TNF-a and RANTES (Figure 8A). Upregulation of MCP-1/CCL2 is interesting since MCP-1/CCL2 is associated with inflammation as well as subventricular zone and neocortical neurogenesis and neurogenic migration [85-90]. MCP-1/CCL2 is expressed by microglia, neurons, neural stem cells and astrocytes [85, 89]. Our data suggest that the increase in MCP-1 protein is unlikely to be microglial-derived since MCP-1 mRNA levels are lower in EOC2 microglia responding to injury than in control microglia (Figure 8B). MCP-1 could be secreted from the increased number of Nestin+ cells. Pluripotent cells have been shown to secrete MCP-1 [85-87, 90] and may contribute to the activation of microglia [88]. Work focused on investigating this possibility is currently underway. Decreased levels of IFN-g and other inflammatory cytokines following co-culture of microglia with injured neurons may also favor neurogenesis in cortical cultures [54, 91]. Inflammatory cytokines are known to act at specific concentrations and in certain combinations to regulate neurogenesis and neuronal survival [29]. More specifically, low levels of IFN-g have been shown to stimulate both neurogenesis and oligodendrogenesis [20, 29, 54, 56]. TNF-a, while primarily associated with inflammatory responses associated with neurotoxicity [29], has varied effects on neurogenesis and can stimulate neurons to secrete CCL2 [89]. Most recent studies show that suppression of TNF-α enhances neurogenesis [18,29,43,75]. MIP-1a and RANTES have diverse roles in the CNS. Various studies have shown that MIP-1a, RANTES, and other ligands binding CCR5 receptors on neurons contribute to pro-inflammatory neurotoxicity [89]. However, these ligands may also play an important role in the development and migration of neurons [29, 50,89]. Interestingly, secretion of pro-inflammatory cytokines by microglia such as IL-1b, IL-6, inducible nitric oxide synthase (iNOS), and TNF-a are suppressed by RANTES signaling [51, 93]. These results suggest that RANTES may function to regulate microglia effector function and contribute to the neurotrophic and neurogenic properties of microglia. Microglia can also secrete growth factors and neurotrophins [39, 61]. Neurotrophin release may be enhanced when microglia respond to cortical injury [94,95]. Other soluble signals such as prokineticins may contribute to the microglial-enhanced neurogenesis presented in this *in vitro* system [77]. We suggest, as have others [18] that microglial effects on neuronal survival, proliferation, and differentiation are largely dependent upon the composition of soluble signals that are released by microglia in response to stimulation. Continued investigations are underway to better dissect the complex milieu of neurogenic soluble signals released by microglia or other secretory cells in the presence of microglia.

Microglial-derived cues have been shown to activate both MAPK and PI3K/AKT pathways while stimulating neurogenesis of cultured progenitor cells and in neurogenic niches [30-38]. For example, IGF-1 secreted by microglia in the hippocampus stimulates neuronal differentiation and activates MAPK and PI3K/AKT intracellular signaling [30-32].In these experiments, only PI3K/AKT signaling was shown to be necessary for microglial-enhanced neuronal differentiation and survival [30-32]. IGF-1 in combination with other growth factors such as TGF-β stimulate neurogenesis and promote cortical cell survival and proliferation via PI3K-p110α [37]. Expression of Nestin during neurogenesis has been shown to require the PI3K pathway but not the MAPK pathway [80]. This study adds to this growing body of work suggesting that the process of neurogenesis requires the activation of PI3K/AKT intracellular signaling. Our results showed that AKT phosphorylation was

increased in cortical cultures following exposure to EOC2 microglial-conditioned media (Figure 9). Application of the PI3K/AKT inhibitor, LY294002, blocks microglial-enhanced neuronal survival and proliferation following injury (Figure 8) and the expression of neurogenic markers (Figure 10). Inhibition of other intracellular signaling pathways associated with viability, proliferation, and neurogenesis such as MEK [96], p38MAPK [84], PKCα/βI/βII/γ [97-99], and Janus Kinase 2 protein [100-101] did not block the increased metabolic activity and viability of cortical cells co-cultured with EOC2 microglia (see supplemental Figure 3S).

Taken together, our data provide an enticing view of the dynamic and multifunctional role of microglia in the cortex. Microglia responding to cortical cues may stimulate local neurogenesis and potential repair after injury. Downregulation of pro-inflammatory cytokine production by microglia could allow for increased proliferation, reduced apoptosis and increased neurogenesis [5,29]. Our results suggest that the microglia enhance neurogenesis and promote neuronal survival by stimulating the PI3K/AKT signaling pathway in cortical cells. While other intracellular signaling pathways are likely also stimulated, the inhibition of the PI3K/AKT pathway and not other pathways previously implicated in neurogenesis blocked EOC2 microglial-enhanced neurogenesis. Further elucidation of the intracellular mechanisms regulating neurogenic function of microglia is essential for understanding the intrinsic neuroprotective role of immune activity in the CNS and may aid in the development of methodologies to promote such activity during neurodegenerative disease or following traumatic injury. The *in vitro* model system presented here provides an experimental tool to investigate the mechanisms of primary microglial responses to cortical injury outside of the neurogenic niche.

Conclusions

Here we present an *in vitro* model system allowing for the assessment of microglial-derived soluble signals on cortical cell viability, proliferation, and differentiation during homeostasis or following cortical injury. Using this model system intracellular signaling pathways are readily investigated in isolated primary cells or cell lines. These specific studies show that cortical cell injury activates neurogenic properties in EOC2 microglia. The specific EOC2 microglial cell line's response to cortical injury results in *at least* the reduction of pro-inflammatory cytokine gene expression and cytokine release. EOC2 microglial-derived soluble signals produced during homeostasis and, more so following activation by acute cortical injury, enhance neurogenesis by upregulating AKT signaling in cortical cells. Increasing our understanding of the mechanisms that drive cortical cell proliferation, differentiation, and survival as stimulated by microglia will provide insight into the neuroprotective role of immune activity in the CNS.

Materials And Methods

Isolation of rat cortical cells

Female timed-pregnant Sprague Dawley rats (200–250 g) were purchased from the Charles River Laboratories (USA). Use of animals was performed in strict accordance with the Institutional Animal Care and Use committee guidelines as approved by the IACUC committee at Creighton University (protocol #0793). Timed Sprague Dawley dams were housed for up to 3 days in Creighton's Animal Resource Facility that is AALAC accredited. Ad libitum food and water and normal 24h light dark schedules were followed. Rat cortical cultures were established as described by Meberg and Miller [57]. Briefly, Sprague-Dawley dams were euthanized by CO_2 asphyxiation. For CO_2 asphyxiation, dam were placed in a clear chamber with CO_2 delivery at 20% of the

chamber volume per minute. After 1 min of cessation of all respiratory movement, toe pinch tests were performed to determine a lack of reflexive responses and a thoracotomy was used to ensure death. E16-E18 rat embryos were removed from their placental sacs and immediately decapitated. The brains were removed and the cerebral cortices were dissected from day 15-16 embryonic Sprague-Dawley rats (Sasco, Wilmington, MA), mechanically dissociated in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution, with 0.035% sodium bicarbonate and 1mM pyruvate (pH 7.4) following 15 min digestion with 2.5% trypsin. Trypsin was neutralized with Dulbecco's Modified Eagles Media (DMEM: Hyclone, Thermofisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum and the cell suspension was washed three times and resuspended with neurobasal media supplemented with B-27® and penicillin/streptomycin (Thermofisher Scientific, Waltham, MA). Cells were then plated onto poly-D-lysine coated plates and coverslips (Sigma, St. Louis, MO) at a density of 1.5 x 10^6 cells/well in 6-well plates and 5 x 10^5 cells/well in 24-well plates and were maintained at 37 ºC in 5% CO_2 in neurobasal supplemented media. Each cortical culture from 1 pregnant dam was considered to be a biological replicate because embryonic brain tissue was used for co-culture, immunocytochemistry, multiplex ELISA, RT-PCR and western blot experiments all performed in triplicate. In total, 24 Sprague Dawley rats were used for these data.

Cultivation of microglia

EOC 2 microglia isolated from brain tissue of *Mus musculus* were purchased from American Type Culture Collection (ATCC CRL-2467; Manassas, VA) and were maintained in DMEM (Hyclone, Thermofisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum, 1% l-glutamine, 1% penicillin/streptomycin, and 20% LADMAC conditioned media. Cells were grown in 100-mm tissue culture dishes at 37 ºC in 5% CO_2 and allowed to reach 80% confluency before the cells were passed. LADMAC conditioned media was collected from *Mus musculus* bone marrow derived LADMAC cells (ATCC CRL-2420; Manassas, VA) 5-7 days after initial plating of cells at 1 x 10^5 in Eagle's Minimal Essential Media (MEM; Hyclone) supplemented with 10% FBS, 1% l-glutamine, 0.1mM nonessential amino acids, 1.0 mM sodium pyruvate, and 1% penicillin/streptomycin. LADMAC conditioned media was collected, filter sterilized, and frozen until needed as a media supplement for microglia. LADMAC conditioned media was used to provide colony-stimulating factor 1 (CSF-1) to the microglial cultures as outlined by the ATCC culture instructions for EOC microglial cells (ATCC CRL-2420; Manassas, VA).

Neuronal – Microglial Co-Cultures

Primary cortical cells were cultured for 48 hours then either injured by mechanical transection using a sterile stylet or left uninjured [74]. Briefly, mechanical transection using a sterile stylet involves the application of the sterile stylet tip directly to the cortical cell culture. Pressure is placed on the stylet while dragging the stylet tip across the cortical cell culture to form parallel sites of injury in the cortical culture. Microglia were pre-seeded directly onto 6-well permeable Transwells® at 5 x 10^5 cells/well or onto 24-well Transwells® at 4 x 10^4 cells/well (Corning,Tewksbury, MA) and cultured for 24 hours before being suspended above cortical cells using Transwells® in the co-culture model system. Microglia seeded onto Transwells® and injured or uninjured (control) primary cortical cells were co-cultured for an additional 48 hours in unsupplemented neurobasal media

prior to cellular assays. For immunocytochemical analysis of EOC2 microglia only, EOC2 microglia were cultured on glass coverslips and placed into Transwells® and then removed for fixing and immunostaining (see below).

Immunocytochemistry

Primary cortical cultures were plated onto sterile poly-D-lysine coated coverslips. Microglia were plated onto sterile glass coverslips suspended above cortical cultures in Transwells®. Following co-culture experiments, cortical cells and microglia were fixed with 4% paraformaldehyde for 15 minutes at room temperature and washed with 1X PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes, washed, and blocked for 1 hour in PBS, 0.2% BSA, and 0.2% Triton X-100. Primary antibodies were applied and incubated overnight at 4 ºC in PBS, 0.2% BSA, and 0.2% Triton X-100. Primary antibodies were purchased from RMD Millipore Sigma (Darmstadt, Germany) and included: mouse anti-Nestin (1:200, Millipore Cat# AB5922, RRID:AB_91107), rabbit anti-GFAP (1:400, Millipore Cat# AB5541, RRID:AB_177521), mouse anti-α-internexin (1:100, Millipore Cat# AB5354, RRID:AB_91800), mouse anti-TUJI/beta tubulin III (1:200, Millipore Cat# MAB1637, RRID:AB_2210524) and mouse anti-NeuN (1:50, Millipore cat#MAB377I, RRID:AB_2298772). Primary antibody directed against neurofilament was a mouse monoclonal neurofilament antibody, p-NF-H (7H11) (1:200, Santa Cruz Biotechnologies, Cat# sc-20015, RRID: AB_670161). To confirm microglial characteristics, microglial cells were immunostained with rabbit anti-mouse CD11b conjugated to Alexa 488 (2 µg/100 µl, Caltag Laboratories, Burlingame, CA). Secondary antibodies were applied for 1 hour at a concentration of 1:500 for goat anti-rabbit IgG (H+L) rhodamine conjugate and goat anti-mouse IgG (H+L) fluorescein conjugate (Pierce, Rockford, IL). Nuclei were visualized using a DAPI stain (300 mmol, MP Biomedicals, Santa Ana, CA). Qualitative and quantitative analysis of immunocytochemistry was performed by acquiring images with a Leica DMI4000B inverted microscope with a cooled CCD camera (Q Imaging, Surrey, BC) and fluorescent capabilities. Quantification of the percent of cells expressing neurogenic markers was determined by counting the number of immunopositive cells for each marker and dividing that number by the total number of cells counted in the field. Experiments were performed in triplicate with at least 300 cells counted manually per experiment for each condition. Quantification of relative fluorescence intensity units (RFU) was calculated by subtracting pixel intensity from the background immunofluorescence of each fluorochrome. Experiments were performed in triplicate with at least 300 cells counted manually per experiment for each condition. In all experiments, images were analyzed with Volocity (PerkinElmer,USA), and ImageQuant (GE Healthcare, USA) software were used for image analysis and presentation. For image data, 3 field views of at least 100 cells from 3 separate experiments were analyzed for each condition.

Measurement of Cell Viability

Viability of cortical cells and microglia were measured by metabolism of thiazolyl blue, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich). Injured and uninjured cortical cells cultured with and without microglia were incubated with 100 µl MTT in 1 ml of media for one hour. Media was removed and cells were dissolved in 300 µl dimethylsulfoxide (DMSO) and aliquoted to 100 µl/well in 96-well plates. Absorbance was read at 540-590nm on an ELISA plate reader. Three experiments were performed in triplicate.

Measurement of Cortical Cell Proliferation

Cell proliferation was measured using Click-iT® EdU Alexa Fluor 647 according to the manufacturer's instructions (C10340, Thermo Fisher Scientific, Waltham, MA). Briefly, Click-iT® EdU Alexa Fluor 647 is a modified thymidine analogue EdU (5-ethynyl-2'-deoxyuridine, a nucleoside analog of thymidine) that is incorporated into newly synthesized DNA. The EdU is fluorescently labeled with a photostable Alexa Fluor® dye during the click reaction. Uninjured and injured cortical cells co-cultured with and without microglia for 2 DIV (days *in vitro*) were fixed in 3.7% formaldehyde in PBS for 15 min at RT. Fixed cells were washed twice with 1 ml of 3% BSA in PBS. Cells were permeabilized in 0.5% Triton®x-100 for 20 min at RT, washed and 1X Click-iT® EdU reaction cocktail was added for 30 min at RT. The reaction cocktail was removed, cells were washed in 3% BSA and PBS, counterstained with DAPI, mounted and imaged for analysis. Imaging was performed using IBIF Leica TCS SP8 MP Confocal Microscope at 20x magnification. Experiments were performed in triplicate with at least 300 cells counted manually per experiment for each condition. Volocity (PerkinElmer,USA) and ImageQuant (GE Healthcare, USA) software were used for image analysis and presentation.

Measurement of Cortical Cell Apoptosis

Apoptosis of cortical cultures was measured following co-culture with microglia for 2 DIV (days *in vitro*) using a Click-iT® TUNEL Alexa Fluor 488 imaging assay (C10245, Thermo Fisher Scientific, Waltham, MA). Manufacturer's instructions were followed. Briefly, injured and uninjured cortical cells cultured with or without microglia were fixed with 4% PFA in PBS for 15 min then permeabilized with 0.25% Triton-X® 100 for 20 min. Each condition was incubated with 100 µl of TdT reaction buffer for 10 min at RT then removed. Cells were incubated in 100 µl TdT reaction cocktail for one hr at 37 ^{o}C. Cells were washed twice in 3% BSA in PBS for 2 min then incubated with Click-iT® reaction buffer with additive for 30 min at RT protected from light. Cells were rinsed and counterstained with DAPI, mounted, and cover slipped for analysis. Imaging was performed using IBIF Leica TCS SP8 MP Confocal Microscope at 20x magnification. Experiments were performed in triplicate with at least 300 cells counted manually per experiment for each condition. Volocity (PerkinElmer,USA) and ImageQuant (GE Healthcare, USA) software were used for image analysis and presentation.

ELISA Analysis

Conditioned media was collected from uninjured and injured neuronal and microglia co-cultures from three separate experiments and cytokine expression was determined by Q-Plex™ mouse cytokine –Inflammation multiplex assay. Concentrations of mouse microglia-derived cytokines MCP-1, IFN-g, MIP-1a, TNFa, RANTES, IL-1a, IL-1b, IL-2, IL-4, IL-3, IL-6, IL-10, IL-12, 1L-17 and GM-CSF were evaluated by Quansys Biosciences (#110449MS, Logan, UT). Cytokine concentrations in media collected from uninjured cortical cells cultured with microglia were used as the reference and control for these experiments. In order to use cytokine concentrations from uninjured cortical and microglial co-cultures as our control condition, each cytokine concentration measured in the uninjured cortical cell and microglia co-culture condition was normalized and set equal to one.

Cytokine concentrations in media collected from injured cortical cells co-cultured with microglia were measured, normalized, and expressed as the percent change in cytokine concentration as compared to uninjured control concentrations for that cytokine. Multiplex ELISA assays were run in triplicate in three biological replicate experiments. Significance of the percent change from control was determined using student T test with Bonferroni correction. The percent change in cytokine concentration was considered significant if $p<0.05$, error bars represent the standard error of the mean of the percent change. Cytokine levels that were not consistently detected in either condition or were not significantly different in control and experimental conditions are not shown.

RT-PCR Analysis

For real-time PCR analysis of cytokines, total RNA of EOC2 microglial cultured on TranswellÒ permeable inserts that were physically separated from cortical cells was extracted using the mirVana miRNA Isolation kit (Ambion). An amount of 200 ng total RNA was reverse-transcribed using the Invitrogen™ NCode™ miRNA First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Comparative real-time PCR was performed using the Invitrogen™ SYBR GreenER™ qPCR SuperMix Universal (Thermo Fisher Scientific) on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. Primers were purchased from QIAGEN (Ccl3, Ccl5, Ifng, Mcpt1, Tnfα, Gapdh). Normalization was performed using GAPDH. Relative expression was calculated using the comparative Ct ($\Delta\Delta$Ct) method.

Western Blots

Following co-culture, protein collected separately from cortical cells or microglia was assessed using western blot analysis. Cortical cells or microglia were lysed with 500 µl lysis buffer (10x lysis buffer, Cat#9803, Cell Signaling, Danvers, MA), supplemented with 0.1M PMSF (Cat # 36978, Thermo Fisher Scientific, Waltham, MA), and HALT™ protease and phosphatase inhibitor diluted to 1X (Cat#78446, Thermo Fisher Scientific, Waltham, MA) per 3 wells of the 6-well plates or per 3 Transwells®. Lysates were spun 10,000 RPM for 10 minutes at 4 °C. Lysate supernatant were collected and heated at 95 °C for 5 minutes with 4X sample buffer plus 10mM DTT. Denatured protein samples were separated by SDS-PAGE gel electrophoresis on 10% TGS gels. Proteins were transferred to PVDF membranes in Tris-glycine transfer buffer. After transfer, membranes were blocked using BSA Blocking Buffer™ in TBS(Cat#37520, Thermo Fisher Scientific, Waltham, MA) for 1 hour and then incubated with primary antibody diluted in BSA Blocking Buffer™ in TBS overnight at 4 °C. Primary antibodies included rabbit anti-phospho-p44/42 MAPK (1:1000, 1:2000, Cell Signaling Technology Cat# 4376, RRID:AB_331772), rabbit anti-pan AKT (1:1000, Cell Signaling Technology Cat# 4691, RRID:AB_915783) rabbit anti-phospho-AKT (1:100, Cell Signaling Technology Cat# 9270, RRID:AB_329824). Following washing in Tris Buffered Saline with 0.1% Tween® 20 and BSA blocking buffer™, appropriate secondary antibodies (anti-rat IgG, HRP-linked antibody,1:1000, Cell Signaling Technology, Danvers, MA) were applied for detection. Membranes were developed using chemiluminescence SuperSignal™ ELISA Pico Chemiluminescent Substrate (Cat#37069, Thermo Fisher Scientific, Waltham, MA) following manufacturer's instructions. Statistical analyses involved semi-quantitative measurements of chemiluminescence using BioRad ChemiDoc QRS (Hercules, CA) imaging

system and software. Total protein loading was assessed by detecting GAPDH in each sample. Three separate experiments were performed for measurements of protein expression by densitometry.

Assays for Intracellular Signal Transduction

Primary cortical and microglial co-cultures were established as described above. Stock solutions of kinase inhibitors in DMSO were prepared at stock concentrations recommended by the manufacturer. Stocks were stored at -20^{o}C and diluted into cell culture media prior to use. Four hours prior to injury and co-culture with microglia, signaling pathway inhibitors were added at concentrations of 0 μM, 10 μM, or 40 μM to the cortical cultures. The following inhibitors were tested: MAPK inhibitor PD98059 (Cat#9900S), PI3K/AKT inhibitor LY294002 (Cat#9901S), PKC and Glycogen synthase kinase-3 inhibitor GF109203X (Cat#984150), Janus kinase 2 inhibitor AG490 (Cat#14704S). All inhibitors were purchased from Cell Signaling Technologies (Danvers, MA). Uninjured and injured cortical cells that were not cultured with microglia were used as controls. For control experiments, DMSO vehicle diluted in culture media was used in the experiments. After 48 hours, cells were analyzed using MTT assays (see above) or fixed with 4% PFA in PBS to observe expression for neurogenic markers, Nestin, α-internexin, and GFAP using immunocytochemical methods as described above. To quantify imaging data, 3 field views of at least 100 cells from 3 separate experiments were analyzed for each condition.

Immunoprecipitation for AKT/pAKT Analysis

Immunoprecipitation for AKT and pAKT was used to increase specificity and detection of AKT protein in cellular lysates. Cellular cultures were lysed as described above and each condition was split into two aliquots (200 μl each). Primary antibodies AKT (pan) (C67E7) rabbit mAB (Cell Signaling Technology Cat# 4691, RRID:AB_915783) and Phospho- AKT (Thr308) rabbit mAB (Cell Signaling Technology Cat#9275, RRID:AB_329828) were added at 1:50 for each sample and rotated overnight at 4 °C. A 50% slurry of EZview Red Protein A Affinity Gel Beads (Cat#P6486, EDM Millipore Sigma, Darmstadt, Germany) were added at 1:10 for each sample and rotated for 1 hour at 4 °C. Cells were centrifuged at 8,200*g* for 1 minute and washed with lysis buffer 3 times. Samples were heated at 95 °C for 5 minutes with 25 μl 3X sample buffer. Samples were run on 4-20% gradient SDS-polyacrylamide gels (Cat#4561096, BioRad, Hercules, CA) using SDS-PAGE and then transferred to PVDF membrane. After transfer, membranes were blocked using BSA Blocking Buffer™ in TBS for 1 hour and then gently rocked with the primary AKT or pAKT antibody at 1:1000 in BSA Blocking Buffer™ in TBS overnight at 4°C. Blots were washed and incubated in secondary anti-rabbit HRP conjugated antibody for 1 hour at RT. Membranes were developed using chemiluminescence as described above. Three separate experiments were performed. Statistical analysis involved analysis of densitometric images acquired with BioRad ChemiDoc QRS imaging system and software (BioRad,Hercules CA) which were performed as described above.

Statistical Analysis

Data are expressed as mean values and error bars represent standard error of the mean (SEM). Student T test with Bonferroni's correction or one- way ANOVA followed by Tukey-Kramer post hoc tests were performed where appropriate. For determination of significant differences between percents and for multiple comparisons between culture conditions, one-way or two-way ANOVA followed by Tukey-Kramer multiple analyses post hoc tests were used. Values of $p<0.05$ were considered to be significant. All statistical analyses were performed with Graphpad Prism 8 (La Jolla, CA).

List Of Abbreviations

AKT: Protein Kinase B, PI3K, phosphatidylinositol 3-kinase, E: embryonic, TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling, NeuN, Fox-3, Rbfox3: Hexaribomucleotide binding protein 3; RT-PCR: Reverse transcriptase polymerase chain reaction, MCP-1: monocyte chemoattractant protein 1, CCL2: chemokine ligand 2 or monocyte chemotactic protein 1, IFN-,g: Interferon gamma, MIP-1a,: macrophage inflammatory protein 1 alpha, TNFa: tumor necrosis factor alpha, RANTES (CCL5): regulation on activation, normal T cell expressed and secreted, chemokine ligand 5, CNS: central nervous system, BDNF: brain derived neurotrophic factor, GDNF: glial derived neurotrophic factor, STAT: signal transducer and activation of transcription, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells, TGF-b: transforming growth factor beta, IGF-1: Insulin growth factor, MAPK: mitogen activated protein kinase, CA1: cornu Ammon 1 of the hippocampus, FGF: Fibroblast growth factor, EGF: epidermal growth factor, IL1-13: Interleukins 1-13, GFAP: glial fibrillary acidic protein, TUJI: neuron specific class III beta tubulin, SEM: standard error of the mean, NF: neurofilament, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, O.D.: optical density, EdU: 5'-ethynyl-2'-deoxyridine, DNA: deoxyribonucleic acid, RFU: relative immunofluorescence units, DAPI: 4',6-diamino-2-phenylindole, DIV: days in vitro, ELISA: enzyme-linked immunosorbent assay, GM-CSF: granulocyte macrophage colony stimulating factor, mRNA: messenger ribonucleic acid, MEK: mitogen-activated protein kinase kinase, p38 MAPK: p38 mitogen-activated protein kinase, PKC: protein kinase C , DMEM: Dulbecco's Modified Eagles Media , FBS: fetal bovine serum, DMSO, dimethylsulfoxide, PBS: phosphate buffered saline, Gapdh: glyceraldehyde 3-phosphate dehydrogenase, ΔΔCt: delta-delta cycle threshold

Declarations

Ethics Approval and consent to participate: Not applicable for humans since there are no human subjects or samples in this study. Use of animals was performed in strict accordance with the Institutional Animal Care and Use committee guidelines as approved by the IACUC committee at Creighton University (protocol #0793).

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests

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Supplemental File Legends

Supplemental Figure Legends

Figure 1. NeuN expression in cortical cells following injury and co-culture with EOC2 microglia. A) Quantification of NeuN+ cells as a percent of the total number of cortical cells. NeuN+ cells were counted in three separate experiments in control conditions, following injury and in co-culture with microglia. A total of at least 300 cells were counted and data are presented as mean with error bars representing SEM. One-way ANOVA was used to determine significance, *p<0.05, **p<0.01, ns is not significant. B) Representative immunofluorescent image of injured cortical cells stained with NeuN and DAPI (for nuclei) 2DIV following injury. C) Representative immunofluorescent image of injured cortical cells co-cultured with microglia and stained for NeuN and DAPI (for nuclei). B-C) The dashed white line indicates the site of injury. The scale bar represents 50 μm.

Supplemental Figure 2. Multiplex ELISA and RT-PCR analyses of inflammatory cytokine protein and mRNA following co-culture with uninjured or injured cortical cells. IL-1a, IL-1b, IL-2, IL-4, IL-3, IL-6, IL-10, IL-12, 1L-17 and GM-CSF were either undetectable in all culture conditions or were not significantly different when detected in co-cultures of uninjured and injured cortical cells with microglia. Error bars represented SEM. Two-way ANOVA followed by Tukey's multiple comparisons test was performed to determine the significance.

Supplemental Figure 3. Inhibitors of MEK, p38, or PKC intracellular signaling pathways do not specifically inhibit EOC2 microglial-enhanced cortical cell viability. A-D) Quantification of MTT viability assays were performed in triplicate experiments using three biological replicates of primary cortical cells. Average OD 595 nm values are shown with error bars representing SEM. Inhibitors were applied at 0, 10, and 40 μM. A) Quantification of viability of uninjured and injured cortical cells alone or in co-culture with microglia in the presence of MEK inhibitor PD98059. B) Quantification of viability of uninjured and injured cortical cells alone or in co-culture with microglia in the presence of p38 MAPK inhibitor SKF86002 C) Quantification of viability of uninjured and

injured cortical cells alone or in co-culture with microglia in the presence of PKCα/βI/βII/γ inhibitor GF109203X. D) Quantification of viability of uninjured and injured cortical cells alone or in co-culture with microglia in the presence of Janus Kinase 2 inhibitor AG490. For each concentration, one-way ANOVA was used to determine significance of the inhibitor. *p,0.05, **p<0.01, ***p<0.001, ****p<0.0001, # indicates that OD values were significantly different from that of control, uninjured cortical cells alone, ns indicates not significant.

Figures

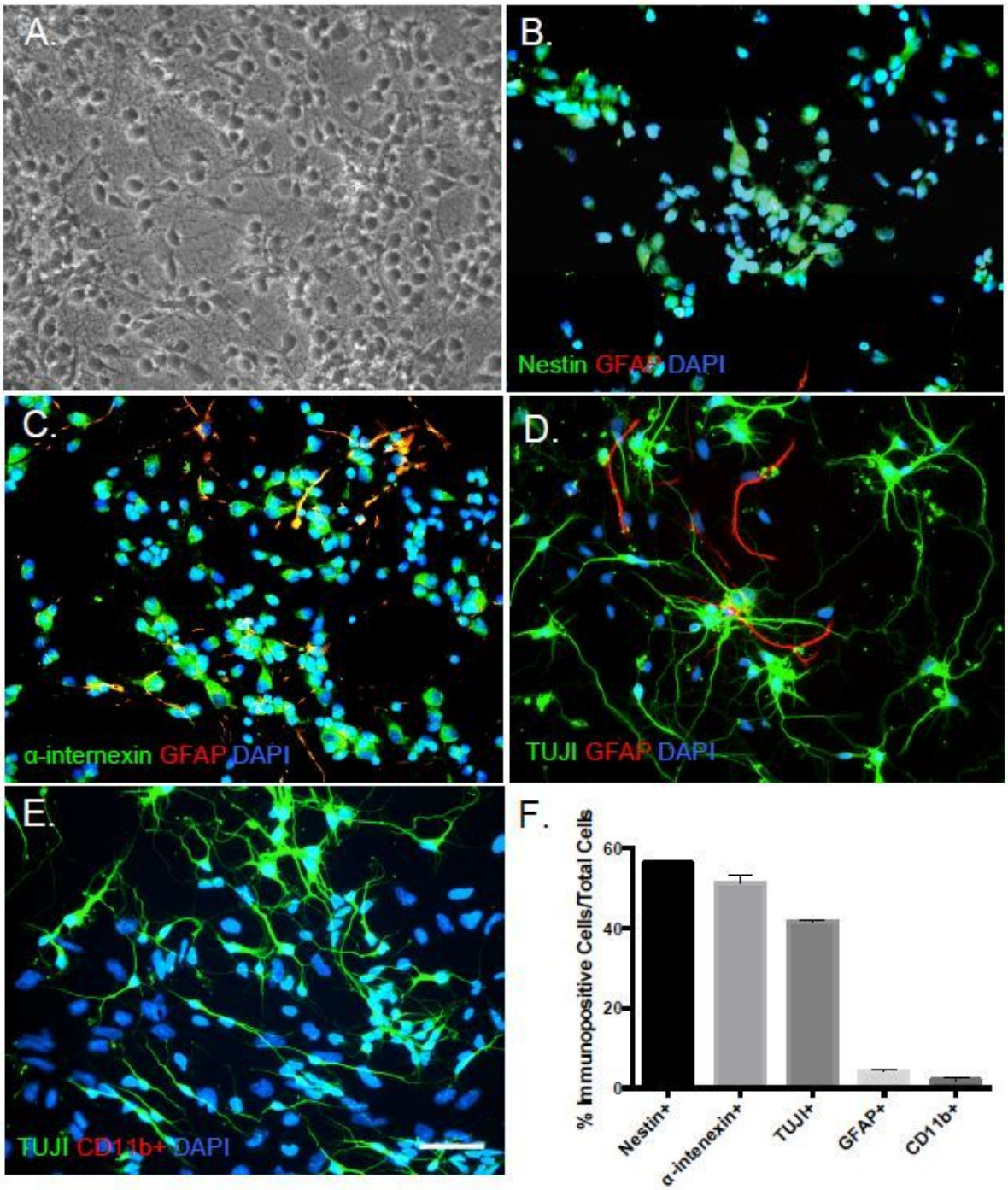
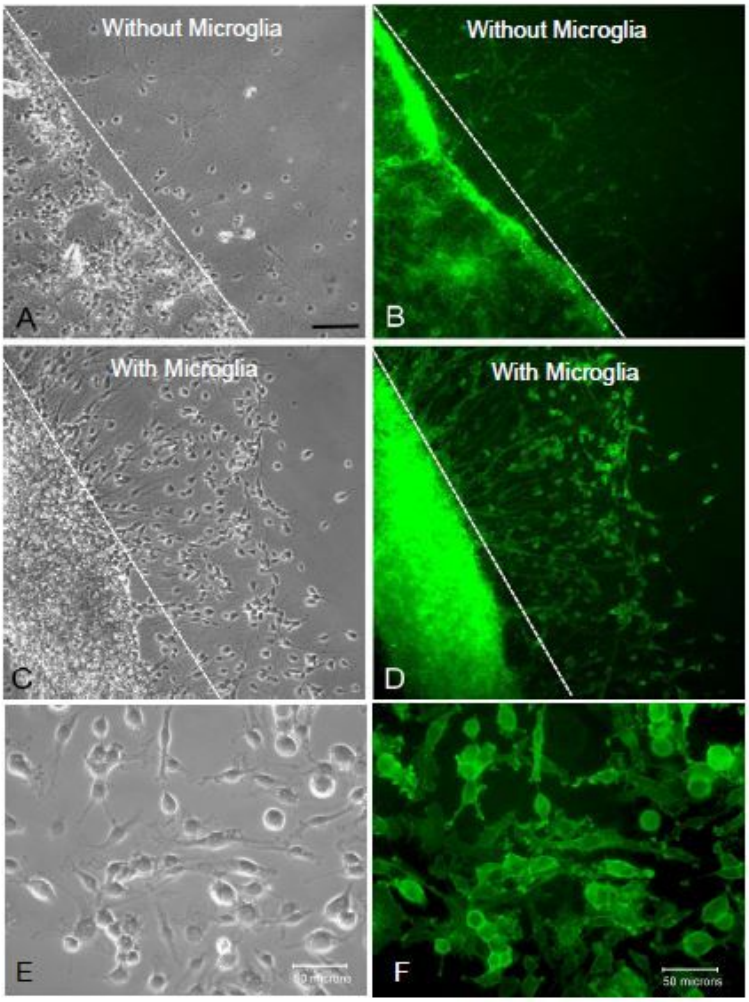


Figure 1

Primary rat cortical cells in the in vitro system. A) Phase contrast image of cortical cells in primary culture. Cells have rounded cell bodies and extension of processes is visible. B) Fluorescent image of primary cortical cells stained for Nestin and GFAP protein expression. C) Fluorescent image of primary cortical cells showing α-internexin+ and GFAP+ cells. D) Fluorescent image of primary cortical cells showing TUJI+ and GFAP+ cells. E) Fluorescent image of primary cortical showing CD11b+ and GFAP+ cells. DAPI (Blue) was to identify nuclei of all cells within an imaged field. Scale bar represents 50 μm and is the same for all images. All images were taken with the 20X Leica objective. F) Quantification of protein expression. The percent of cells immunopositive for each marker compared to the total number of cells was calculated for three separate fields with each field having at least 100 cells or over 300 cells total. Error bars represent SEM.



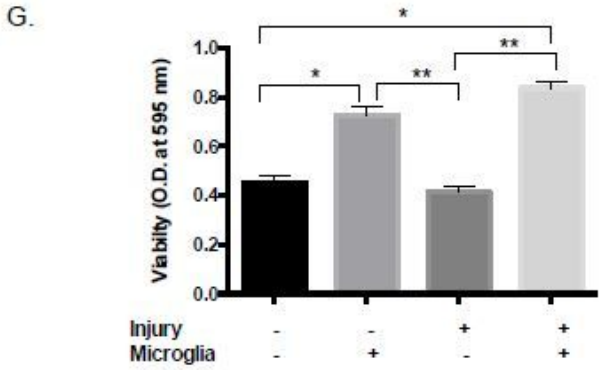


Figure 2

Phase contrast and fluorescent images showing the effect of microglial co-culture with primary cortical cells following injury in the in vitro system. A). Phase contrast image of primary cortical cells following injury mediated by stylet transection. The dashed white line indicated the site of injury. Two days in vitro (2DIV) following injury a few cells can be observed beyond the injury site. Black scale bar represents 100 µm. B) Fluorescent image of NF+ cortical cells 2DIV following injury. NF+ immunoreactivity indicated the location of neuronal progenitors and neurons in primary cortical cultures following injury and 2DIV without microglia. C) Phase contrast image of primary cortical cells following injury and co-culture with microglia for 2DIV. An increase in the number of cortical cells at and beyond the site of injury is visible (dashed white line represents the site of injury). D) Fluorescent image of NF+ cortical cells in microglia co-culture 2DIV following injury. Dashed white indicates the site of injury. A-D). Images were taken with the 20X Leica objective. E) Phase contrast images of cultured activated microglia cells co-cultured with primary neurons on Transwell® inserts. F) Microglia used in this co-culture system are immunopositive for the microglial marker CD11b-Alexa 488 (green immunofluorescence) antigen. E-F) Scale bar represents 50 µm. Images were taken with the 40X Leica objective. G) Viability of uninjured cortical cells or injured cortical cells with or without microglial co-culture for 2DIV was assessed using the MTT assay. MTT activity was read at OD 595 nm for three separate experiments. Data show average OD at 595nm with error bars representing SEM. One-way ANOVA with multiple comparisons were performed to determine the significance of viability data. Significance is *p<0.05, ** p<0.01.

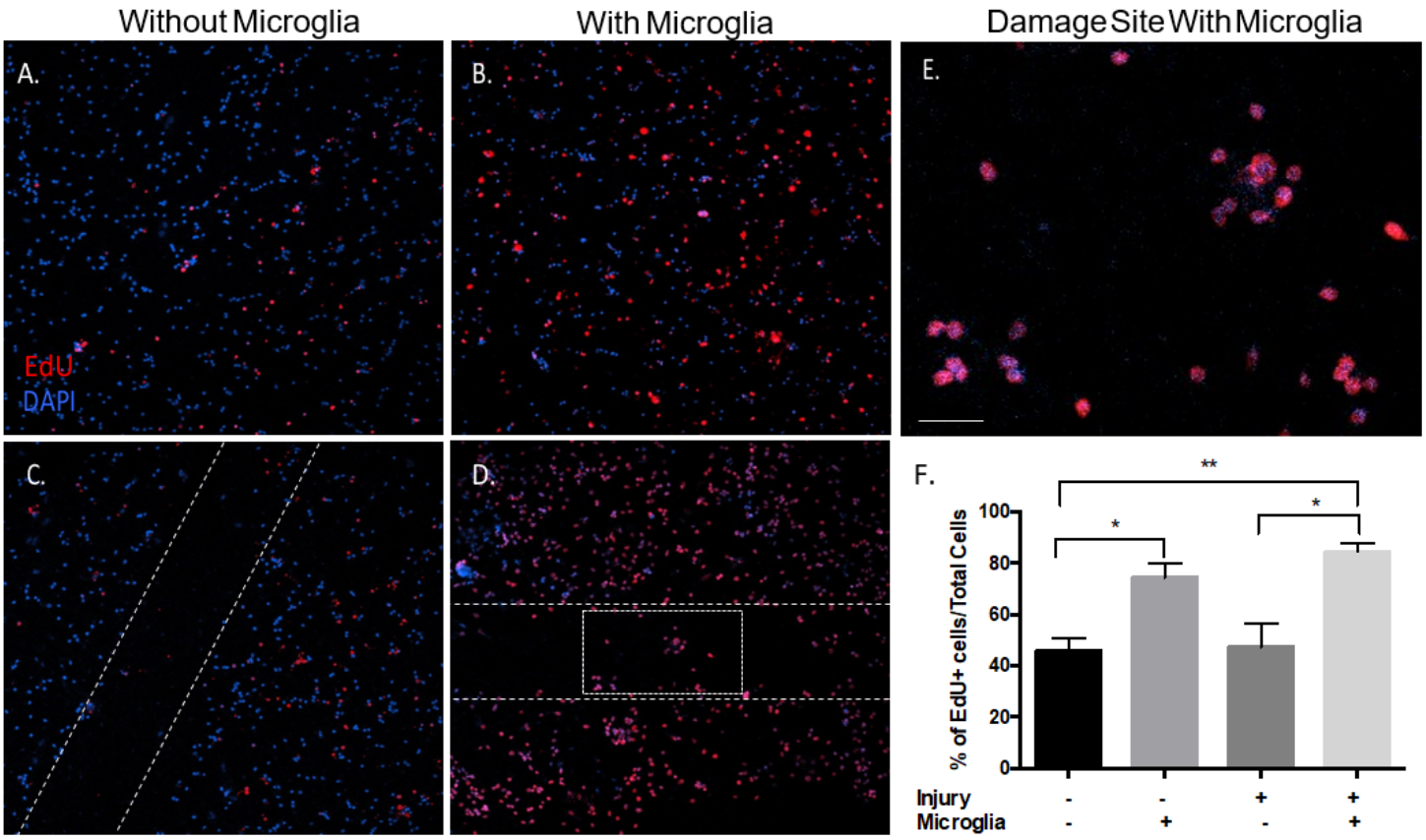


Figure 3

EOC2 microglial co-culture increased proliferation of uninjured and injured cortical cells. A) Click-iT® EdU Alexa Fluor 647 (red) immunofluorescent staining of uninjured cortical cells cultured in the absence of microglia. B)

Click-iT® EdU Alexa Fluor 647 immunofluorescent staining of uninjured cortical cells co-cultured with microglia. In the presence of microglial co-culture, EdU+ cells increased. C) Click-iT® EdU Alexa Fluor 647 immunofluorescent staining of injured cortical cells cultured in the absence of microglia. Injury is indicated by the dashed white line. D) Click-iT® EdU Alexa Fluor 647 immunofluorescent staining of injured cortical cells co-cultured with microglia. Injury is indicated by the dashed white line. The site of injury shown in E) is indicated by the dashed white rectangle. E) Full magnification of the injury site (dashed rectangle in D) showing EdU+ cells within the site of injury. Hoechst immunofluorescence (blue) indicates nuclei (A-E). All images were taken with the Leica confocal using the 20 X objective. Scale bar represented 100 µm and applies to A-D) where E) shows full magnification and partial view of the imaging field. F). Quantification of EdU+ primary cortical neurons. The percent of EdU+ cells in each experiment was calculated from the total number of cells counted. Cells were identified by using Hoechst to immunostain nuclei (Blue). In three experiments, 300 or more cells in each field were counted for each condition. Error bars represent SEM. One-way ANOVA followed by Tukey's multiple comparisons test was performed to determine the significance. Significance is *p<0.05, ** p<0.01.

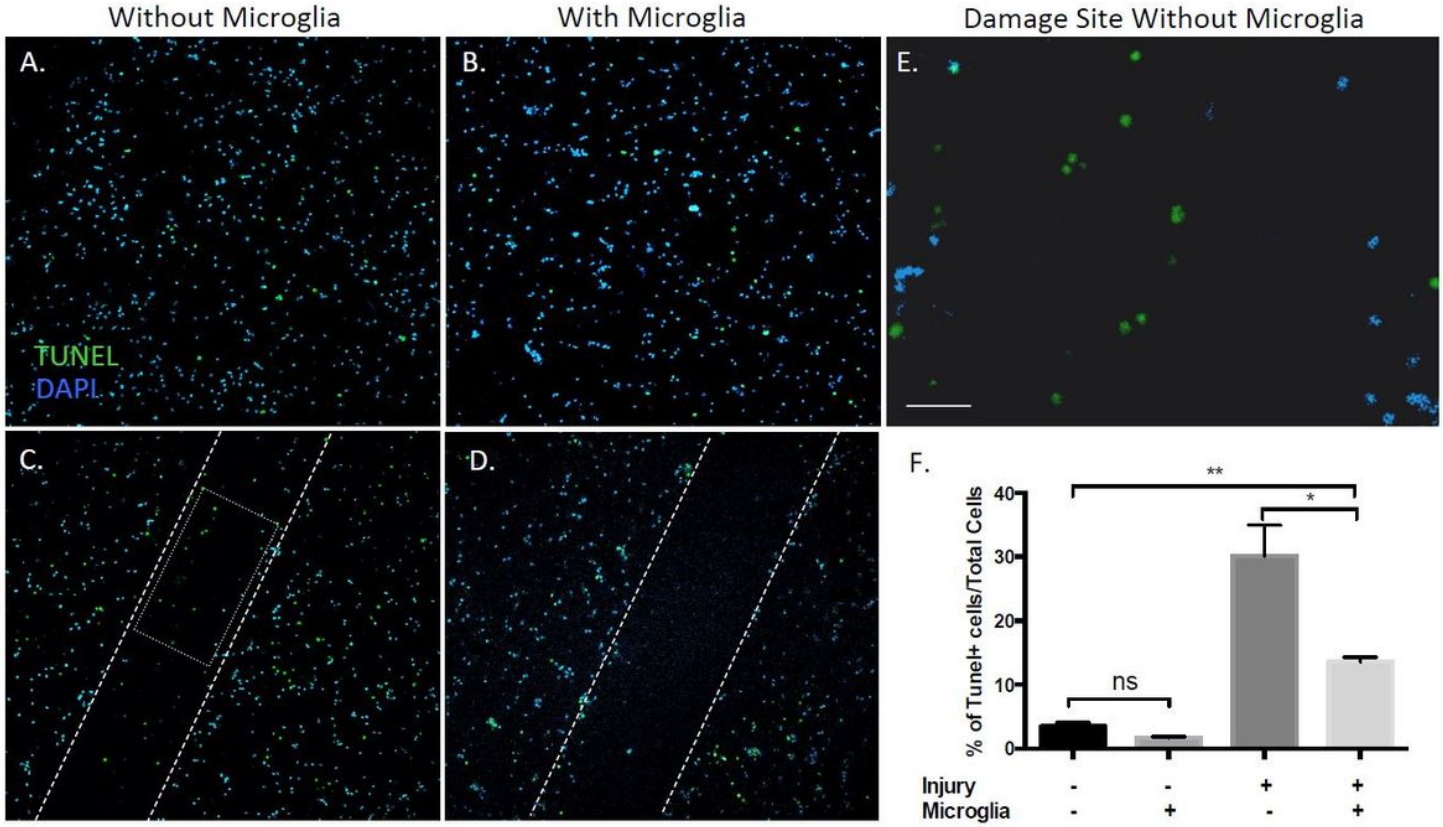


Figure 4

Microglial co-culture reduced apoptosis of injured cortical cells. A) Click-iT® TUNEL Alexa Fluor 488 (green) immunofluorescent staining of uninjured cortical cells cultured in the absence of microglia. B) Click-iT® TUNEL Alexa Fluor 488 immunofluorescent staining of uninjured cortical cells co-cultured with microglia. C) Click-iT® TUNEL Alexa Fluor 488 immunofluorescent staining of injured cortical cells cultured in the absence of microglia. At the site of injury and beyond, TUNEL+ cells were present. D) Click-iT® TUNEL Alexa Fluor 488 immunofluorescent staining of injured cortical cells co-cultured with microglia. A reduction of TUNEL+ cells at the site of injury and beyond was noticeable and significant (F). Hoechst immunofluorescence (blue) indicates

nuclei A-D. All images were taken with the Leica confocal using the 20 X objective. Scale bar represented 100 µm and applies to A-D) where D) shows full magnification and partial view of the imaging field. F) Quantification of TUNEL+ primary cortical cells. The number of TUNEL+ cells was compared to the total number of Hoechst immunostained nuclei. In three experiments, 300 or more cells in each field were counted for each condition for quantification. One-way ANOVA with multiple comparisons were performed to determine the significance of viability data. Significance is *p<0.05, ** p<0.01, ns is not significant.

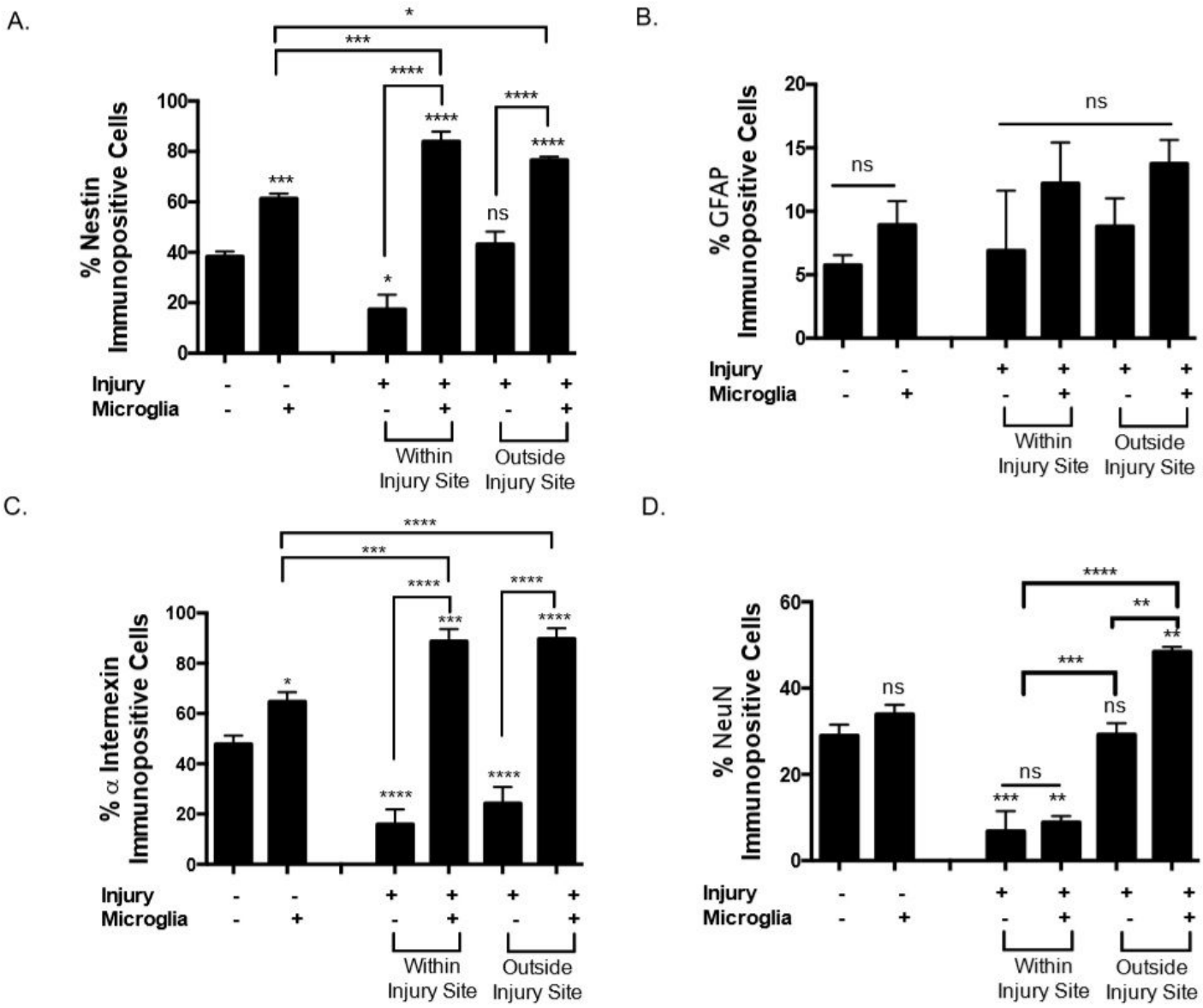


Figure 5

EOC2 microglia increase the percent of cortical cells expressing neurogenic markers, particularly within the site of injury. The percent of cells Nestin+ (A), GFAP+ (B), α-internexin+ (C), and NeuN+ (D) immunopositive cells was determined by comparing the number of immunopositive cells to all cells that were counted in each culture condition. Cells were identified by using Hoechst to immunostain nuclei. Cortical cells cultured alone served as control. Uninjured and injured cortical cells were cultured alone or in the presence of EOC2 microglia suspended on Transwells®. Quantification of immunocytochemical data within the injury site and outside the injury site is shown. Error bars represent SEM. Two-way ANOVA followed by Tukey's multiple comparisons test was

performed to determine the significance. Significance is *p<0.05, ** p<0.01,***p<0.001, *****p<0.0001, ns is not significant.

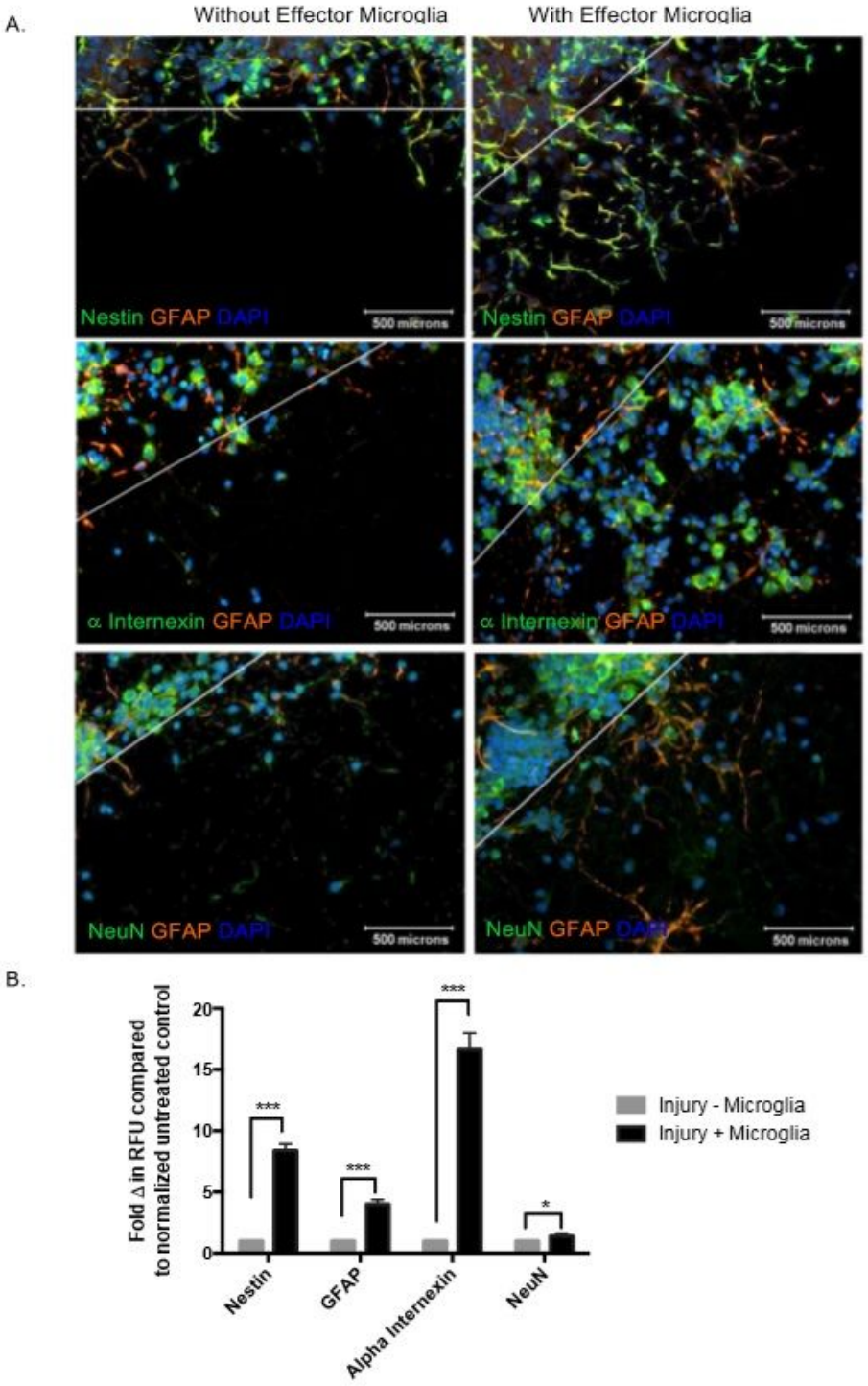


Figure 6

EOC2 microglia stimulate increased expression of neurogenic markers in injured cortical cell co-cultures. A) Immunofluorescence of Nestin+ (green), α-internexin+ (green), NeuN+ (green) and GFAP+ (red) cells in injured neurons co-cultured with control or microglial conditioned media. DAPI (blue) was used to observe nuclei of all cultured cells. White line indicates the site of injury. Scale bar represents 500 μm. All images were acquired with a 20X Leica objective. B) Three separate fields within injured neuronal cultures were evaluated for protein

expression using immunofluorescent measurement software to determine the fluorescence intensity units for each protein marker. Averaged fluorescent intensity data from injured cortical cell cultures were normalized and set equal to 1 to determine relative fluorescent intensity units (RFU). Fold change in RFU in injured cortical cultures with microglia was determined and multiple Student T Tests were performed to determine significance. Error bars represent SEM. Significance is **p<0.01, *** p<0.001, **** p<0.0001.

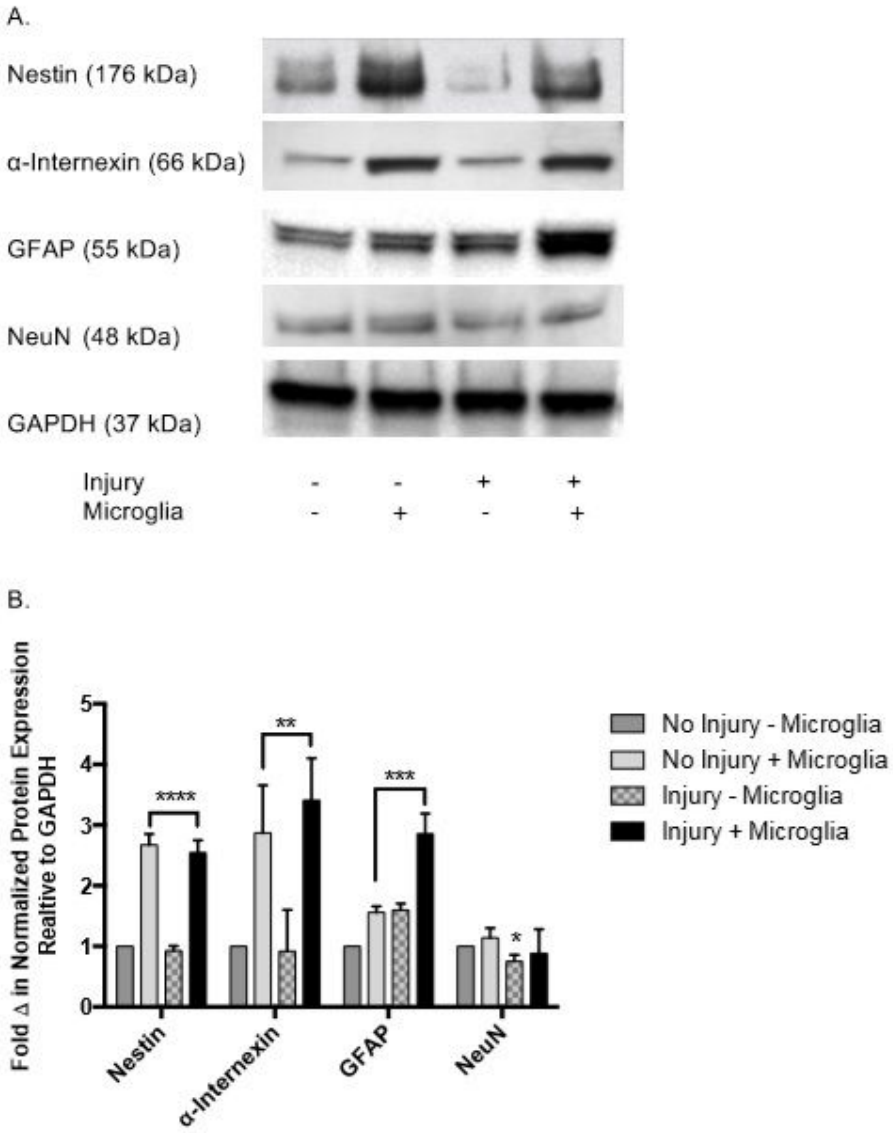


Figure 7

Western blot analysis of Nestin, α-internexin, NeuN, and GFAP expression in uninjured and injured neuronal cultured exposed to EOC2 microglia or control media. A) Representative western blot images of protein from uninjured neuronal cultures in control media without microglia, uninjured neuronal cultures in microglial-conditioned media, injured neuronal cultures in control media without microglia, and injured neuronal cultures in microglial- conditioned media. GAPDH was used as a total protein loading control. B) Quantification of relative protein expression in western blot experiments. Experiments were run in triplicate using primary cultures from three biological replicates. Error bars represent SEM. Two-way ANOVA followed by Tukey's multiple comparisons test was performed to determine the significance. Significance is *p<0.05, ** p<0.01,***p<0.001, *****p<0.0001.

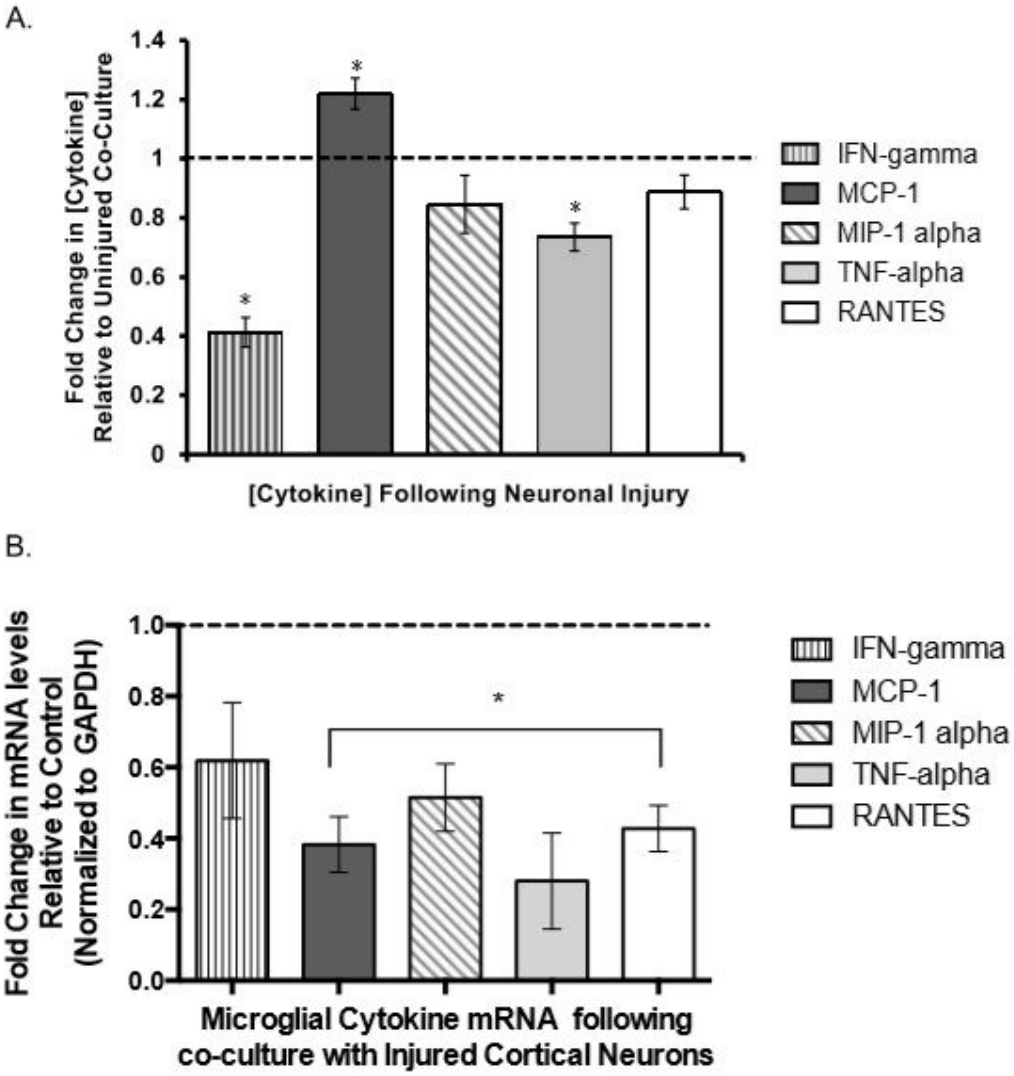


Figure 8

Multiplex ELISA and RT-PCR analyses of inflammatory cytokine protein and mRNA following co-culture with uninjured or injured cortical cells. A) Relative cytokine levels in media collected from injured cortical cell and microglial co-culture as measured by multiplex ELISA assays are shown. The dashed line represents the normalized cytokine levels for IFN-γ, MCP-1, MIP-1α, TNF-α, and RANTES in media collected from co-cultures of EOC2 microglia and uninjured cortical cells. Normalized uninjured co-culture cytokine concentrations were set equal to one. The experimental data represent the average fold change in each cytokine as measured in the media collected from injured cortical cell and EOC2 microglial co-culture. Experiments were run in triplicate from three biological replicates. Error bars represent SEM. Students T Test was used to determine whether the fold change was significance as compared to normalized control, Significance is *p<0.05. B) qRT-PCR analysis of cytokine mRNA levels in EOC 2 microglia following stimulation with injured cortical cells. EOC2 mRNA was collected from microglia suspended above cortical cultures on Transwells®. Fold change in mRNA levels was normalized to Gapdh expression in EOC2 microglia following stimulation with injured cortical cells. Fold change is compared to mRNA in EOC2 microglia co-cultured with uninjured neurons. Control mRNA expression is indicated by the dashed line set at one. MIQE guidelines were followed. Mouse specific primers were used for

qRT-PCR analysis of mouse microglial cells. Experiments were run in triplicate for three biological replicates. Error bars represent SEM. Significance was determined using BioRad CFX Manager software, *p<0.05.

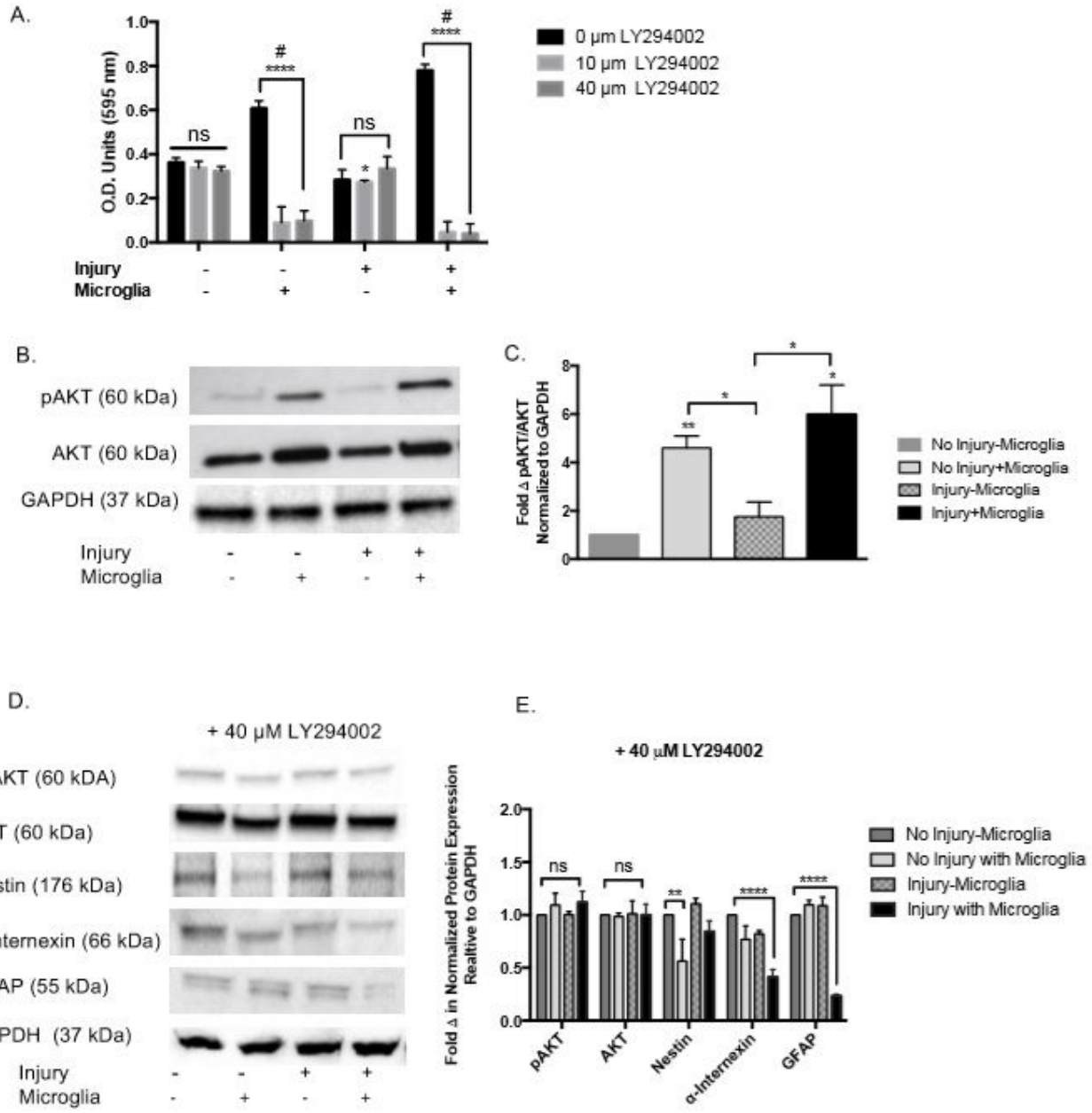


Figure 9

Effect of PI3K inhibition microglial-enhanced cortical cell viability and AKT phosphorylation in cortical cells following EOC2 microglial co-culture. A) Quantification of MTT viability following LY294002 treatment of uninjured and injured cortical cells alone and in microglial co-culture. For each concentration, one-way ANOVA was used to determine significance effect of the inhibitor. Significance is *p<0.05, **p<0.01, ***p<0.001, at p<0.05, ****p<0.0001, # indicates that OD values are significantly different from that of control, uninjured cortical cells alone, ns indicates not significant. Application of the MAPK inhibitor, PD98059 (10 µM and 40 µM) did not significantly reduce microglial enhanced mitochondrial activity in uninjured or injured co-culture experiments. Mitochondrial activity of neurons co-cultured with microglia remained significantly higher than that of neurons alone. B) Representative western blots illustrating pAKT phosphorylation in injured and injured cortical cultures with and without microglial co-culture. Culture conditions treated with 0 µm and 40 µm are

shown. C) Quantification AKT phosphorylation as compared to total AKT protein levels normalized to GAPDH. Three separate western blot experiments were analyzed, data were averaged and error bars represent SEM. One-way ANOVA was used to compare the significance the data for 0 μm and 40 μm LY294002 treatments. Significance is *p<0.05, **p<0.01.

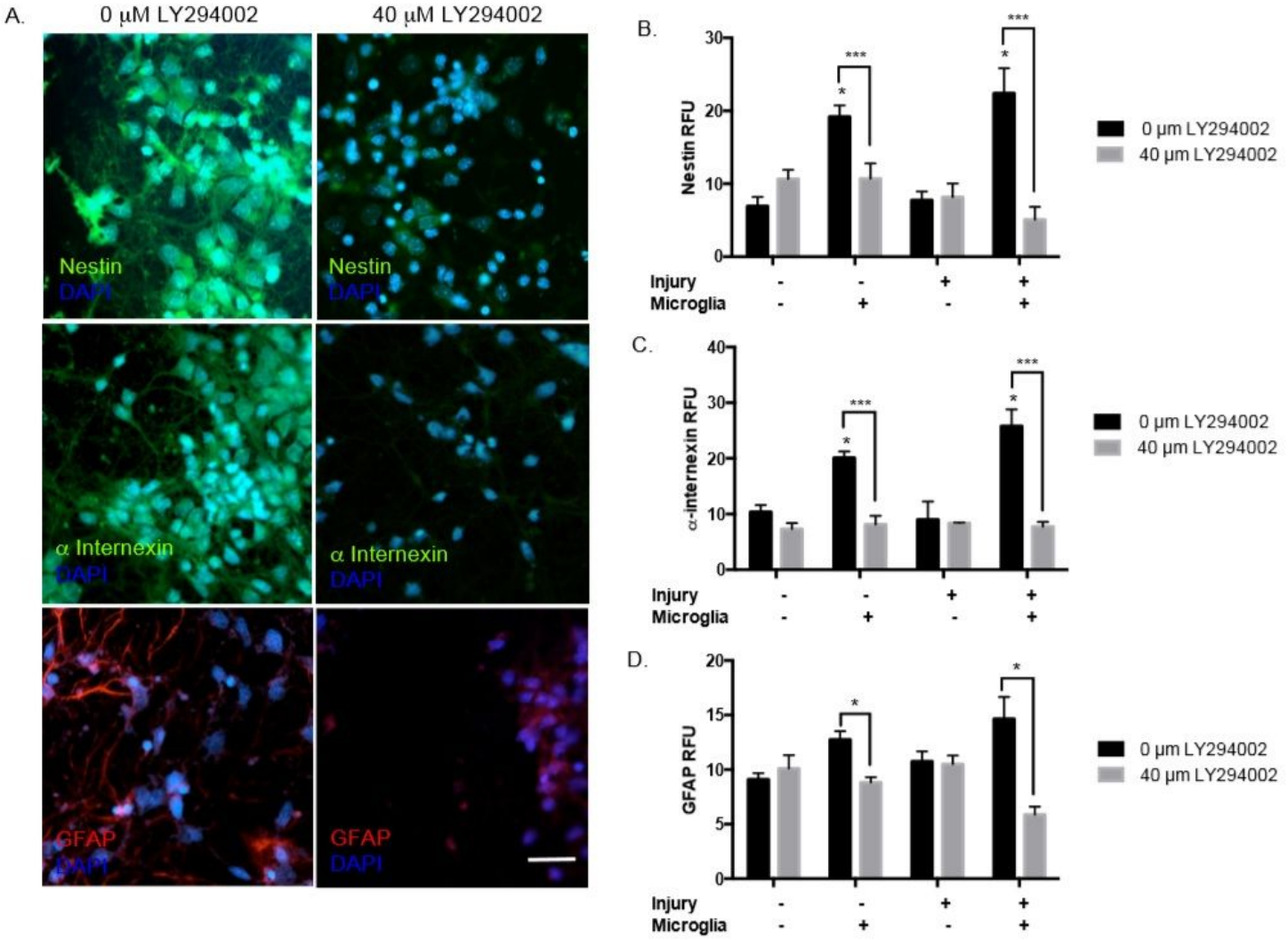


Figure 10

Inhibition of PI3K blocks microglial-enhanced expression of neurogenic markers in injured cortical co-cultures. A) Immunofluorescence of Nestin (green), α-internexin (green), or GFAP (red) and DAPI (blue to indicate nuclei) in injured neurons co-cultured with microglia in 0 μM or 40 μM LY294002. Application of 40 μM LY294002 significantly reduced Nestin, α-internexin, and GFAP immunofluorescence. DAPI (blue) was used to observe nuclei of all cultured cells. All images were acquired with a 40X Leica objective. Scale bar represents 50 μm. B) Quantification of immunofluorescence for neurogenic markers. Three separate fields within uninjured and injured cortical cultures that were treated with 0 μM or 40 μM LY294002 and stained for each neurogenic marker were evaluated using immunofluorescence measurement software. Fluorescence for each marker is shown as a relative fluorescence intensity unit (RFU). Data represent the average RFU's for the three fields. Error bars represent SEM. Student's t-test were used to determine the significance of LY294002 treatment in each

condition. One-way ANOVA was used to compare treatments and microglial co-culture to uninjured cortical control cells. Significance is *p<0.05, **p<0.01, ***p<0.001.

Supplementary Files

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