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

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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 of the classic neurogenic niches. We used an in vitro co-culture model system to test the hypothesis that microglia respond to soluble signals from injured cortical cells to alter the cortical environment in order to promote cortical neurogenesis and maintain cell survival. Results Our model system allows for assessment of how microglial soluble signals influence mechanically injured cortical cells in vitro. These data demonstrate that microglia responding to soluble signals from uninjured and, to a greater extent, injured cortical cells enhanced cortical cell viability and proliferation. Co-culture of injured cortical cells with microglia significantly reduced apoptosis as shown by TUNEL immunocytochemistry. Microglial-derived soluble cues enhanced the proliferation of cells expressing neurogenic markers nestin, glial fibrillary acidic protein, and α-internexin as determined by western blot and immunocytochemistry. Significantly increased NeuN expression was observed in injured cortical cultures co-cultured with microglia. Multiplex ELISA assays and RT-PCR analysis revealed significant increase of MCP-1/CCL2 and downregulation of IFN-γ, MIP-1α, TNFα and RANTES in media from microglial and injured cortical co-cultures compared to uninjured controls. Microglia soluble cues increase AKT phosphorylation in cortical cells particularly following injury. Inhibition of AKT phosphorylation in cortical cells blocked the microglial-enhanced cortical cell viability and expression of neurogenic markers in vitro. Conclusion An in vitro model system allows for assessment of microglial-derived soluble signals, independent of cell-cell contact, on cortical cell viability, proliferation, and differentiation during homeostasis or following injury. While many intracellular signaling pathways may be activated in neurons by neurogenic microglial-derived soluble cues, these data suggest that microglial-derived soluble signals produced during homeostasis and following activation by acute cortical injury enhance neurogenesis by upregulating AKT signaling. Increasing our understanding of the mechanisms that drive cortical neurogenesis stimulated by microglia during homeostasis and following injury will provide insight into neuroprotective role of immune activity in the 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 to 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].

During homeostasis and following injury, microglia support neurogenesis in the classic neural stem cell niches in the dentate gyrus of the hippocampus and subventricular and subgranular zones of the lateral ventricle [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 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 via 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-, MIP-1α and RANTES/CCL5, IL-1α, and IL-1β suppresses apoptosis and enhances neurogenesis [29]. Taken together, a complex combination of microglial-derived soluble environmental cues with neurogenic or neuroinflammatory functions likely work in combination to promote or restrict neurogenesis and survival during development and into maturity [18].

Outside the classic neural stem cell niches, CNS stem cells have the potential to generate neurons and glial cells following ischemia or traumatic injury [43-48]. Microglial-derived soluble cues are potentially important for local endogenous neurogenesis and neuronal survival. Interestingly, microglial invasion of the cortical plate overlaps with peak periods of cortical neurogenesis [1-3]. After invasion, microglia remain as morphologically and functionally dynamic cells within the environment of the cortex [2-3]. Consequently, we hypothesize that microglia are able to 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 microglial respond to cortical cues to regulate neuronal differentiation and survival during homeostasis and following acute mechanical injury. Our *in vitro* system utilized a microglia cell line suspended above primary rat cortical cells that are injured or left uninjured as control. Primary cortical cells are isolated from Sprague-Dawley rat embryos at day 16-18 because this species, at this age, has been well shown to yield a high population of mammalian cortical cells that can be grown *in vitro* and can provide a tool to study neuronal survival and differentiation [49]. Our data suggest that microglial-derived soluble cues promote cortical cell viability and enhance proliferation of cortical cells. In these studies, microglial derived cues increased viability and proliferation and reduced apoptosis of primary cortical cells following acute mechanical injury *in vitro*. Increased expression of neurogenic markers Nestin, GFAP, and α-internexin is seen in cortical cultures following co-culture with microglia. Expression of the mature neuronal marker NeuN increased in injured cortical cells co-cultured with microglia. Microglia

responding to acute injury downregulate their expression of pro-inflammatory cytokines. AKT phosphorylation is increased in cortical cells co-cultured with microglia and blocking AKT phosphorylation reduces the enhanced expression of neurogenic markers. These results show that the co-culture *in vitro* system provides a model system to further evaluate cortical cell responses to microglia activation and to investigate the underlying mechanisms of the functional states of microglia in response to cortical signals during homeostasis or following injury. This system may provide valuable insight into mechanisms that should be evaluated for relevance *in vivo* and the potential for stimulating neuroimmune interactions that favor recovery after cortical injury.

Results

Cortical and microglia co-cultures were established to investigate the effect of microglial responses to homeostatic and injury signals from cortical cells *in vitro.* Primary cortical cultures were established using methods previously described [49]. To characterize the cortical cell types at the time of microglial co-culture, immunocytochemical analysis of cortical cell marker expression was performed at two days *in vitro* (Figure 1). In the control cortical culture, 56.3 ± 0.3% of cells express Nestin, 51.3 ± 2.0% express α-internexin, 41.7 ± 0.3% of cells express the mature neuronal marker TUJI, and 4.3 ± 0.3% express 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 microglia 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 microglia, cortical cells were injured using a sterile stylet to disrupt and remove cortical cells from the culture plate. Injured cortical cells were then cultured for two additional days with or without microglia on Transwell® inserts. Two days post-injury, in cortical cultures without microglia, the site of injury (indicated by the dashed white line) is observable and few neurofilament immunopositive (NF+) cells or processes are found in the injury site (Figure 2A, B; black scale bar represents 100 µm). In cortical cultures with microglia, the site of injury (indicated by the

dashed white line) is associated with increased cell density and increased neurofilament expression at the site of injury and in the area of clearance (Figure 2C, D). Microglia 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 microglial 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 microglia and uninjured cortical cultures with and without microglia 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 neuronal mitochondrial activity by 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 microglia 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 microglia 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 microglia (Figure 2G). Following injury, metabolic activity as a measure of cell viability was not significantly different from MTT activity in uninjured control conditions (Figure 2G, $p>0.05$, n=3,).

Immunocytochemistry 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 microglia. 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 microglia showed the presence of EdU+ cells demonstrating that these cultures have at least a limited number of dividing cells upon isolation from the cortex (Figure 3A). In the presence of microglia, the number of EdU+ cells increased in uninjured cortical cell culture (Figure 3B). Mechanical injury of cortical cells striped away cortical cells as indicated by dashed white lines (Figure 3C-D). Without microglia, few EdU+ cells were in the damaged area (Figure 3C). When injured cortical cells were co-cultured with microglia, 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 proliferating cells in uninjured cortical culture without microglia showed that 45.7 ± 5.0% of the cells were EdU+. In the presence of microglia, 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 microglia was significant (Figure 3E, *$p < 0.05$, ± represents SEM, n=3). Following injury, the percent of EdU+ cells in cortical cultures without microglia was 47.2 ± 9.3% and was not significantly different from the control, uninjured cortical cells cultured without microglia (Figure 3F, $p>0.05$, ± represents SEM, n=3,). When cultured with microglia, the number of proliferating EdU+ cells in injured cortical cultures increased 38.5 ± 6.0% to 84.3 ± 3.3% compared to uninjured control cells (Figure 3F, **$p<0.01$, ± represents SEM, n=3).

To evaluate the effect of microglia 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 microglia. In the absence of microglia, very few TUNEL+ cells were present in uninjured cortical cell cultures (Figure 4A). Following injury, cortical cells in the absence of microglia, showed increased TUNEL expression particularly in the area of damage (Figure 4C). When injured cortical cells were co-cultured with microglia, 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 microglia shows that 1.72 ± 0.2% of the cells were TUNEL+. Co-culture of microglia 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 microglia significantly increased to 30.1 ± 4.9% (± represents SEM, n=3, *$p<0.05$, Figure 4F). When cultured with microglia, the number of TUNEL+ cells in injured cortical cultures decreased to 5.6 ± 1.2% (± represents SEM, n=3, Figure 4F). The microglial-induced reduction of TUNEL staining in injured cortical cultures by 24.6 ± 4.8% was significant (**$p<0.01$, ± represents SEM, n=3, Figure 4F). The percent of TUNEL+ cells in injured cortical cultures in the presence of microglia 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 TUNEL expression was reduced and EdU expression was increased in injured cortical cells co-cultured with microglia, we sought to determine whether these surviving and proliferating cells were undergoing neurogenesis. Progressive expression of Nestin, glial fibrillary acidic protein (GFAP), and α-internexin, and NeuN is indicative of stages of neurogenesis [51-54]. Immunocytochemical analysis was used to observe the expression of Nestin, glial fibrillary acidic protein (GFAP), and α-internexin, and NeuN immunoreactivity following injury and exposure to microglia. Relative immunofluorescence units (RFU) of immunocytochemistry experiments were measured to assess the level of protein expression in all culture conditions. 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 marker, over 100 cells per image of three culture fields were averaged and compared to controls in three separate experiments (Figure 5). Following injury, cortical cells were cultured in the presence of microglia or, for untreated controls, in media alone. Immunofluorescent images for Nestin and GFAP or α-internexin and GFAP, or NeuN and GFAP showed increased expression of these neurogenic and neuronal markers in injured areas when co-cultured with microglia as compared to media alone. Nuclei for all cells are identified with DAPI (blue immunofluorescence). Nestin and GFAP expression was significantly enhanced in injured neuronal cultures co-cultured with microglia as compared to cortical cells cultured alone (Figures 5A). Cells immunopositive for both Nestin (green) and GFAP (red) are indicated by yellow immunofluorescence. A few Nestin+/GFAP+ cells were observed in injured areas when cortical cells were cultured in control media (Figure 5A). Nestin immunofluorescence increased 7.4 ± 0.3fold in injured cortical cultures exposed to microglia-conditioned media as compared to Nestin immunofluorescence in injured cortical cultures without microglia (± represents SEM, n=3, ****$p<0.0001$, Figure 5B). Expression of α-internexin, significantly increased 16.7 ± 0.8fold in injured cortical cultures when exposed to microglial- conditioned media as compared to untreated injured controls (± represents SEM, n=3, ****$p <0.0001$, Figure 5B). Overall a 4.0 ± 0.2fold increase in GFAP immunofluorescence was observed in injured cortical cultures co-cultured with microglia as compared to controls (± represents SEM, n=3 ***$p<0.001$, Figure 5B). NeuN staining was 0.4 ± 0.1fold higher in injured cortical cultures with microglia than without at 2 DIV (± represents SEM, n=3 **$p<0.01$ Figure 5B). In control, uninjured cortical cultures the percent of NeuN+ cells was 35 ± 1.5% at 2DIV (± represents SEM, n=3, see supplemental data, Figure 1SA). The presence of microglia did not significantly increase the percent of NeuN+ cells by 2 DIV in uninjured cultures (Figure 1SA, $p>0.05$). Injury of cortical cultures reduced the percent of NeuN+ cells to 28.3 ± 0.3% which was significantly different from uninjured controls (± represents SEM, n=3 Figure 1SA-B, **$p<0.01$). The percent of NeuN+ cells in injured cortical co-cultures increased to 44 ± 0.6% when co-cultured with microglia. This increase was significant when compared to injured cortical cultures cultured without microglia and

control conditions (*p<0.05, Figure 1SB). These data suggest that microglia responding to injury signals from cortical cells can increase neuronal differentiation of cortical progenitor cells.

Western blot analysis was used to assess protein expression of neurogenic markers in injured as well as uninjured cortical cultures (Figure 6A). Nestin protein expression in uninjured and injured cortical cultures increased 1.7 ± 0.1fold and 1.5 ± 0.1fold respectively following co-culture with microglia as compared to expression in uninjured control cultures alone (± represents SEM, n=3, ****p<0.0001, ***p <0.001, Figure 6B). No significant change in Nestin expression was observed in cortical cultures that were injured and not co-cultured with microglia (p>0.05, n=3, Figure 6B). A 1.9 ± 0.5fold increase in α-internexin expression was observed in uninjured cortical cultures co-cultured with microglia as compared to control, uninjured neurons cultured alone (± represents SEM, n=3, **p <0.01, Figure 6B). Injured cortical cells co-cultured with microglia exhibited a 2.4 ± 0.4fold increase in α-internexin expression compared to control, uninjured neurons cultured without microglia (± represents SEM, n=3**p <0.01, Figure 6B). No significant change in Nestin expression was observed in cortical cultures that were injured and not co-cultured with microglia (p>0.05, n=3, Figure 6B). GFAP, a protein expressed during neurogenesis and in mature astrocytes, levels increased 1.6 ± 0.1fold in uninjured cortical cells co-cultured with microglia and 1.9 ± 0.2fold in injured cortical cells co-cultured with microglia as compared to control uninjured cortical cells (± represents SEM, n=3, ***p <0.001, Figure 6B). Expression of GFAP also significantly increased ~1.6 fold in injured cortical cells without microglial co-culture (***p<0.001, n=3, Figure 6B). Expression of the mature neuronal marker NeuN in uninjured or injured cortical cells co-cultured with or without microglia for 2 DIV was evaluated (Figure 6, and Supplemental Figure 1). Injury of cortical cells followed by 2 DIV without microglial co-culture reduced NeuN expression significantly by 0.25 ± 0.1fold 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 microglia was not significantly different that NeuN expression in control conditions as determined by western blot (± represents SEM, n=3, p>0.05, Figure 6B).

In order to examine more specifically the soluble signals released by microglia in the co-culture system, multiplex ELISA assays were used to determine the presence of well-characterized microglial-derived cytokines in microglial-conditioned media following injury of co-cultured cortical cells and then compared to the cytokine levels in microglial-conditioned media following co-culture with uninjured cortical cells. Analysis of three separate assays performed in triplicate showed that the concentration of several cytokines was significantly different from the levels observed in microglia-conditioned media when suspended above uninjured cortical cells (Figure 7). 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 TNFa 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 7A). 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 7B). IL-1a, IL-1b, IL-2, IL-4, IL-6, and GM-CSF were not significantly different in conditioned media from uninjured and injured cortical and microglial co-cultures (data not shown).

RT-CPR was used to compare cytokine mRNA levels in microglia co-cultured with injured neurons to mRNA levels in control microglia co-cultured with uninjured neurons. Microglia 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%) TNFa (decreased by 97.6 ± 4.1%) and RANTES (decreased by 62.5 ±11.6%) (± is SEM, Figure 7B). Decreased expression of MCP-1 mRNA in microglia suggests that the increase in MCP-1 protein levels was not microglial derived (Figure 7B). Given that microglia can secrete a variety of soluble factors with signaling capabilities, studies directed at identifying key components of microglial soluble signals, particularly in co-cultures with injured cortical cells 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 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 microglia 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 2SA-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 microglia. AG490 and GF109203X at 40 µm did significantly influence viability of cortical cultures but these affects were not specific for microglial-enhanced viability (Figure 2S). 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 40 µm respectively as compared to untreated, uninjured cortical cells co-cultured with microglia (± represents SEM, n=3, ****$p < 0.0001$, Figure 8A). Following injury, 10 and 40 µm 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 8A). Treatment of uninjured or injured cortical cells in the absence of microglia with LY294002 did not significantly affect metabolic activity as measured by MTT (± represents SEM, n=3, $p > 0.05$, Figure 8A). Western blot analysis of cortical cells confirmed that the presence of microglia in suspension above cortical cultures increased phosphorylation of AKT, a PI3K target, in cortical cells (Figure 8B). Analysis of western blots showed that the presence of microglia 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 8C). Injury alone did not significantly increase (~0.8 fold) phosphorylation of AKT as compared to control levels (Figure 8C, $p > 0.05$). Following injury and co-culture with microglia, AKT phosphorylation increased 5.0 ± 1.0fold as compared to injured cortical cells alone (±

represents SEM, n=3, **p <0.01, Figure 8C). 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 8C). These experiments suggest that phosphorylation of AKT may be necessary for microglial-enhanced neurogenesis.

To further investigate the necessity of the AKT phosphorylation for microglial-enhanced neurogenesis, immunocytochemical analysis of neurogenic protein expression was assessed in injured and uninjured cortical cells co-cultured with microglia in the presence of 40 mm LY294002. Uninjured cortical cells incubated with LY294002 but not microglia 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 seen in cortical cells when cultured with microglia (Figure 8B). Western blot analysis indicated that application of LY294002 reduced microglial-enhanced protein expression of Nestin, a-internexin, and GFAP in injured cortical cultures as compared to controls (Figure 6A-6B and Figure 8D-8E). LY294002 reduced Nestin expression by 0.44 ± 0.3fold in uninjured cortical cells co-cultured with microglia as compared to controls (**p<0.01, n=3, Figure 8E). LY294004 treatment reduced Nestin expression by 0.26 ± 0.0fold in injured cortical cells co-cultured with microglia as compared to uninjured controls (***p<0.001, n=3, Figure 8E) and by 0.36 ± 0.1fold in injured cortical cells cultured without microglia (**p<0.01, n=3, Figure 8E). 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 microglia when compared with uninjured controls and by 0.40 ± 0.1fold (**p<0.01) when compared with injured cortical cells not cultured with microglia (Figure 8E, n=3). Additionally, microglial-enhanced expression of GFAP in uninjured and injured cortical cells was significantly reduced following treatment with LY294002 (Figure 8D and 8E). 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 8E).

Immunocytochemical analysis was also used to evaluate the effect of blocking PI3K activity and AKT phosphorylation (Figure 9). 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 9A) since the expression of these markers was enhanced in our co-culture system. DAPI immunofluorescence was used to identify nuclei (Figure 9A). Immunocytochemical analysis confirmed 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 microglia (Figure 9A). 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 (Figure 5). Evaluation of RFU for Nestin expression showed that co-culture with microglia significantly enhanced Nestin in uninjured or injured cortical cultures as compared to Nestin expression in cortical cultures alone (*$p<0.05$, Figure 9B). Application of 40 mm LY294002 reduced Nestin expression in co-cultures of microglia and uninjured cortical cells by 8.5 ± 2.6 RFU or ~44% (***$p<0.001$, ± is SEM, n=3, Figure 9B). In co-cultures of microglia and injured cortical cells, 40 mm LY294002 reduced Nestin expression by 17.3 ± 3.9 RFU or ~77% (***$p<0.001$, ± is SEM, n=3, Figure 9B). LY294002 application did not significantly affect Nestin expression in cortical cells that were not cultured with microglia ($p>0.05$, n=3, Figure 9B). 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 microglia (Figure 9C). 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 9D). 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 microglia to support the viability, proliferation, and neurogenesis 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 [48, 55-56]. Cortical microglial cells are a potential source of neurogenic signaling molecules [4, 39-42, 45-47]. 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, 45-47]. 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, 57-58]. Several *in vitro* studies suggest that microglial-conditioned media is neurotoxic depending on the mechanism of microglial activation [57-59].

Results from experiments presented here show that soluble cues from microglia responding to injury signals from primary cortical cells enhanced proliferation, suppressed apoptosis, and promoted the expression of neurogenic and mature neuronal markers in the primary cortical cultures. In the absence of microglia, the cortical cells in this *in vitro* system 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). Immunocytochemical analysis and MTT assays of cortical cell and microglial co-culture following injury showed that microglia enhanced viability of neurofilament immunopositive cells and increased extension of neurofilament (heavy and light) immunopositive processes into injured areas of cortical cultures (Figure 2). Neurofilament is expressed in neuronal progenitor cells and mature neurons. Neurofilament expression is associated with structural maturation of neurons and axonal function as light, medium, and heavy neurofilaments assemble [60]. Our immunocytochemical data show that microglia responding to injury release soluble signals that increase heavy and light neurofilament expression in viable cortical cells *in vitro*. Inflammatory responses of microglia are

associated with reduced neurofilament expression, disrupted axon development, and demyelination [61-62] although the role of microglia-derived inflammatory response in axon degeneration and regeneration in the visual system has recently been called into question [63]. Given our current understanding, increased expression of neurofilament in these *in vitro* studies suggests a suppression of proinflammatory responses in microglia.

To more specifically address how microglia responding to cortical injury affect cortical cells, 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 microglia significantly increased the percent of EdU positive cells in cortical cultures as compared to controls (Figure 3). EdU positive, or proliferating cells were observed in the area of damage and throughout the culture (Figure 3). Microglia 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, 45]. In the subgranular zone and subventricular zones the microglial responses are dependent upon location and specific developmental time points [4-5, 46-47]. Apoptosis as measured by TUNEL staining showed that co-culture with microglia significantly reduced apoptosis in cortical cells following injury (Figure 4). Microglia can increase or reduce 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 responding to cortical damage may be activated, at least at specific times following injury, to attract differentiating neuronal progenitors into cortical tissue [64]. These studies suggest microglia may also act locally to stimulate neurogenesis of responsive cortical cells at noncanonical neurogenic regions [48, 65, 69]. We propose that this *in vitro* model system is useful to investigate whether microglial soluble signals following cortical injury are neurogenic outside of previously investigated subventricular and subgranular zones.

Microglial-conditioned media increased expression of neurofilament immunopositive cells and processes following cortical injury (Figure 2) so further investigation of expression of neurogenic markers, Nestin, a-internexin, and GFAP was warranted (Figures 5). Quantification of protein expression using immunofluorescence and western blot analysis showed significant differences in expression levels for these markers when injured neurons were co-cultured with microglia. Nestin expression alone or Nestin and GFAP expression are characteristic protein expression patterns of early, primary neurogenic progenitors in classic neurogenic regions [2,44,65,66]. Nestin immunopositive progenitors can eventually give rise to intermediate progenitors that produce immature neurons or neuron-committed progenitors [65-66]. These microglial-derived soluble cues may also enhance gliogenesis [67-70]. Nestin and GFAP expression are associated with the proliferation of migratory astrocyte progenitors following trauma in the neurogenic niche of the subependymal zone [53, 68]. Enhancement of Nestin expression in injured cortical cultures following exposure to microglial-derived soluble cues presents a potential mechanism by which local signaling may stimulate the process of neurogenesis following injury in the cortex outside of classic neurogenic regions [69]. The expression of a-internexin, a Type IV neuronal intermediate filament protein occurs during later stages of neuronal differentiation and axon development [54, 60]. Immunocytochemical and western blot results showed that a-internexin expression was significantly increased in injured neurons following co-culture with microglia (Figures 5-6). Neuronal progenitor cells expressing a-internexin were clearly distinct from GFAP expressing cells. The over two-fold increase in expression of a-internexin in injured cortical cultures exposed to microglial-conditioned media suggests that proliferating cells were neuronal progenitors (Figures 5-6). Quantification of western blot experiments also showed a significant increase in a-internexin in injured cortical cultures exposed to microglial-conditioned media as compared to uninjured controls (Figure 6). Immunocytochemical analysis of GFAP immunoreactivity in injured cortical cultures co-cultured with microglia showed an increase in GFAP expressing cells with the morphology of mature astrocytes as compared to injured cortical neurons alone (Figure 5). Western blot analysis confirmed a significant increase in GFAP protein in injured cortical cells cultured with microglia (Figure 6). It is likely that microglial

responding to injury stimulate neurogenesis and GFAP expression in progenitor cells as well as some mature astrocytes. Microglial soluble signals have been shown to stimulate astrocyte differentiation from progenitor cells [71]. In order to determine whether microglia 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 [52, 55]. Enhanced NeuN immunofluorescence was observed in injured cortical cultures exposed to microglial-conditioned media (Figures 5, 1S). The presence of NeuN positive cells in injured areas may represent microglial-directed neuronal differentiation of newly generated neuronal progenitor cells. Few of these 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 microglia (Figure 6A-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 time and given the expression pattern neurogenic markers observed in these experiments we are likely observing primarily early stages of neurogenesis. Long-term experiments are underway to determine whether maturation of mature neurons results from effector microglial-enhanced neurogenesis. Our supplemental data showing increased NeuN immunofluorescence in the area of injury following cortical co-culture with microglia, suggests that microglial-enhanced neurogenesis and differentiation of mature neurons is occurring (Figure 1S). Taken together, these data suggest that microglial soluble signals released following activation by cortical injury promote the proliferation and survival of neurogenic cells and that these soluble signals can give rise to mature neurons and astrocytes necessary for cortical repair and normal function.

While continued studies are necessary to identify the specific injury signals that stimulate microglia neurogenic properties, our results showed the early neurogenic

potential of cortical cells when exposed to microglial-derived soluble signals. Multiplex ELISA assays of microglial-conditioned media from co-culture experiments with uninjured and injured cortical cells revealed that the expression of several microglial-derived cytokines significantly changed following 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 7A). IL-1a, IL-1b, IL-2, IL-4, IL-6, and GM-CSF were detected in our co-culture conditions, however, the concentration of these cytokines was not significantly different in microglial co-culture with uninjured or injured neurons and are unlikely contributors to microglial-enhanced neurogenesis observed in this study (data not shown). 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 [72-75]. MCP-1/CCL2 is expressed by microglia, neurons, neural stem cells and astrocytes [76]. Our data suggest that the increase in MCP-1 protein is unlikely to be microglial-derived since MCP-1 mRNA levels are lower in microglia responding to injury than in control microglia (Figure 7B). MCP-1 may be secreted from the increased number of Nestin immunopositive cells as pluripotent cells have been shown to secrete MCP-1 [76,77] and may contribute to the activation of microglia and microglia-derived neurogenesis [52]. 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 neuronal cultures [49]. 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, rather than high levels produced during responses to inflammatory mediators, have been shown to stimulate both neurogenesis and oligodendrogenesis [29, 70, 78]. TNF-a, while primarily associated with inflammatory responses associated with neurotoxicity [29], has varied effects on neurogenesis and can stimulate neurons to secrete CCL2 [73]. Most recent studies show that suppression of TNF-α enhances neurogenesis [29, 79]. Similarly, MIP-1a and RANTES have diverse roles in the CNS. Various studies have shown that MIP-1a, RANTES, and other ligands for CCR5 receptors on neurons contribute to pro-inflammatory neurotoxicity [80]. However, these

ligands may also play an important role in the development and migration of neurons [46,80]. Interestingly, secretion of pro-inflammatory cytokines by microglia such as IL-1ß, IL-6, inducible nitric oxide synthase (iNOS), and TNF-a are suppressed by RANTES signaling [47, 81]. 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]. Neurotrophin release may be enhanced when microglia respond to cortical injury [82,83]. Additional ELISA and RT-PCR analyses are required to determine whether neurotrophins or other soluble signals, such as prokineticins, contribute to the microglial-enhanced neurogenesis presented in this *in vitro* system [64]. We suggest, as have others [18] that microglial effects on neuronal survival, proliferation, and differentiation is largely dependent upon the entire composition of the mixture of soluble signals that are released by microglia in response to stimulation.

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 [31-38]. For example, IGF-1 secreted by microglia in the hippocampus stimulates neuronal differentiation and can activate MAPK and PI3K/AKT intracellular signaling but only PI3K/AKT signaling is necessary for 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 [66]. 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 microglial-conditioned media (Figure 8). 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 9). Inhibition of other intracellular signaling pathways associated with viability, proliferation, and neurogenesis such as MEK [84], p38MAPK [62], PKCα/βI/βII/γ [85-87], and Janus Kinase 2 protein [88, 89] did not

block the increased metabolic activity and viability of cortical cells co-cultured with microglia (see supplemental Figure 2S).

Taken together, our data provide an enticing view of the dynamic and multifunctional role of microglia in the cortex. Microglia responding to cortical cues, particularly following injury, may stimulate local neurogenesis and may impact cortical function and potential repair after injury. Downregulation of pro-inflammatory cytokines release from microglia could allow for increased proliferation, reduced apoptosis and increased neurogenesis [5,29]. Continued investigations are underway to better dissect the complex milieu of neurogenic soluble signals released by microglia. Our results suggest that in combination the microglial-derived soluble cues enhance neurogenesis 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 such as MEK, p38 MAPK, PKCα/βI/βII/γ and Janus Kinase 2 blocked 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 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 cortical cells and/or microglia. These studies show that cortical injury activates neurogenic properties in microglia that involve *at least* the reduction in pro-inflammatory cytokine gene expression and cytokine release. Microglial-

derived soluble signals produced during homeostasis and following activation by acute cortical injury enhance neurogenesis by upregulating AKT signaling in cortical cells. Increasing our understanding of the mechanisms that drive cortical neurogenesis stimulated by microglia during homeostasis and following injury will provide insight into 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 Faculty 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 [49]. 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 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 sacks 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 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 is used for co-culture, immunocytochemistry, Multiplex ELISA, RTPCR 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 [50]. 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 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.

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, 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#MAB377l, 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. Images were analyzed with ImageProPlus software (MediaCybernetics, Rockville, MD). 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. Briefly, 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. Volocity (PerkinElmer,USA) and ImageQuant (GE Healthcare, USA) software were used for image analysis and presentation. Experiments were performed in triplicate with at least 300 cells counted per experiment for each condition.

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 for15 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. Volocity (PerkinElmer,USA) and ImageQuant (GE Healthcare, USA) software were used for image analysis and presentation. Experiments were performed in triplicate with at least 300 cells counted per experiment for each condition.

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 and mature miRNAs, total RNA 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 (ΔΔCt) 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 include 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 -20ºC 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. Inhibitors tested were the following: 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) 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. Values of $p<0.05$ were considered to be significant. All statistical analyses were performed with Graphpad Prism 6 (La Jolla, CA).

List Of Abbreviation

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 Figure Legends

Supplemental Figure 1. NeuN expression in cortical cells following injury and co-culture with 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. Inhibitors of MEK, p38, or PKC intracellular signaling pathways do not specifically inhibit 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 are significantly different from that of control, uninjured cortical cells alone, ns indicates not significant.

Figures

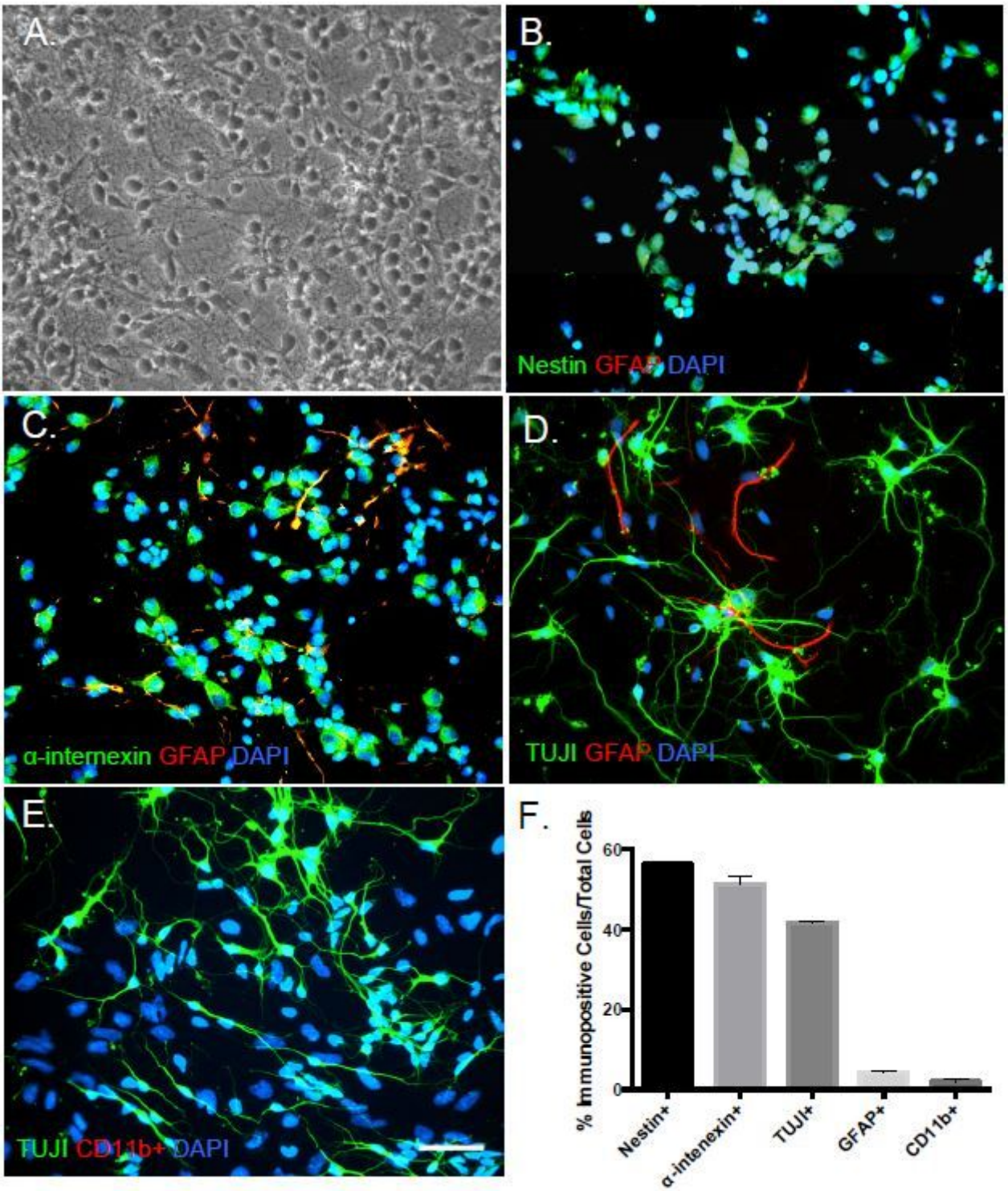
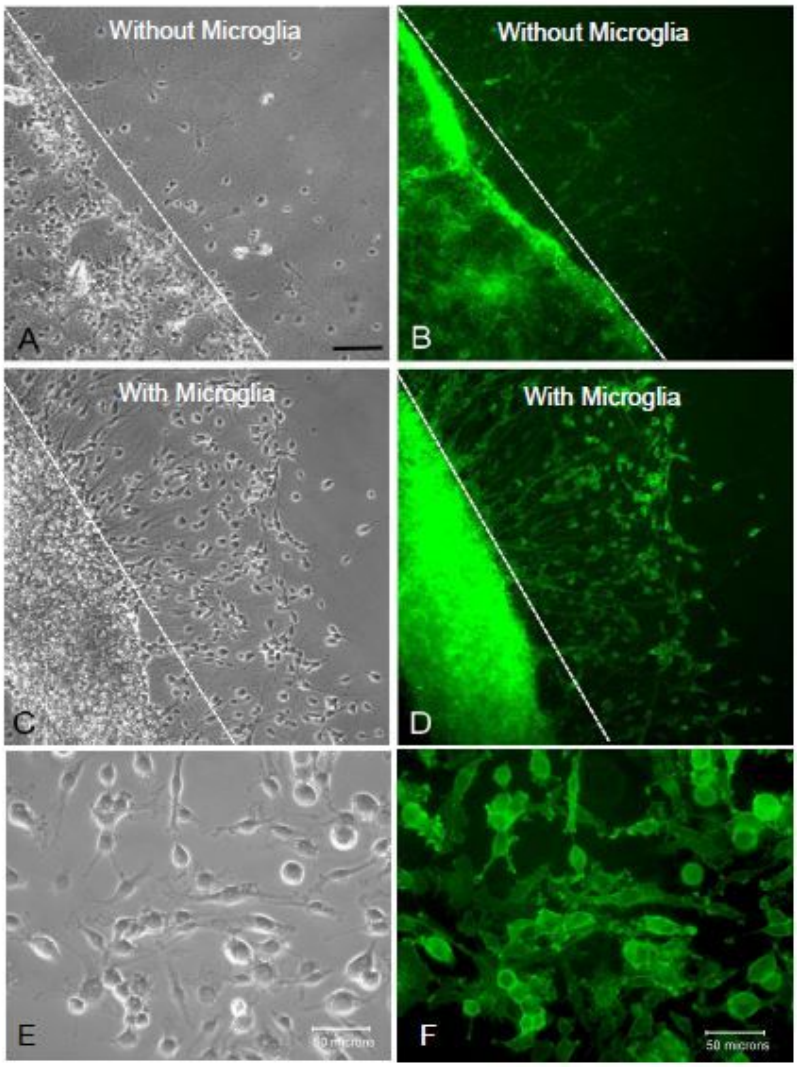


Figure 1

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.



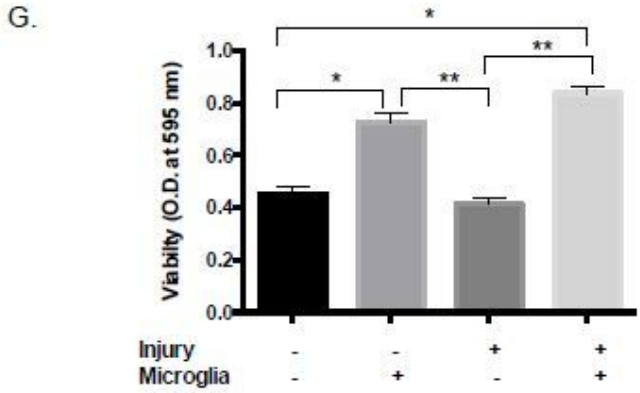


Figure 2

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.

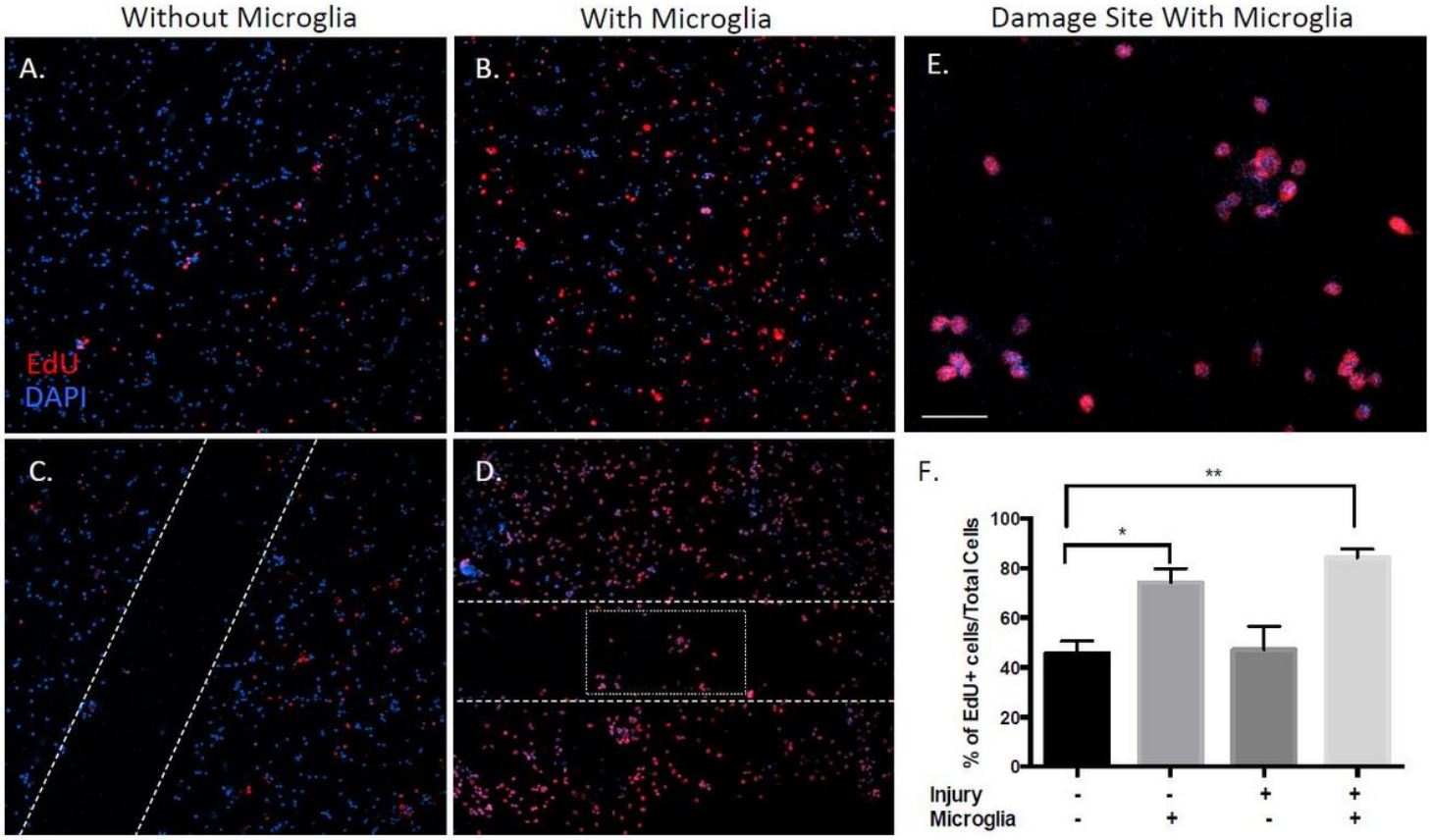


Figure 3

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 an increase in EdU+ cells was visible. 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 with 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 number of EdU+ 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.

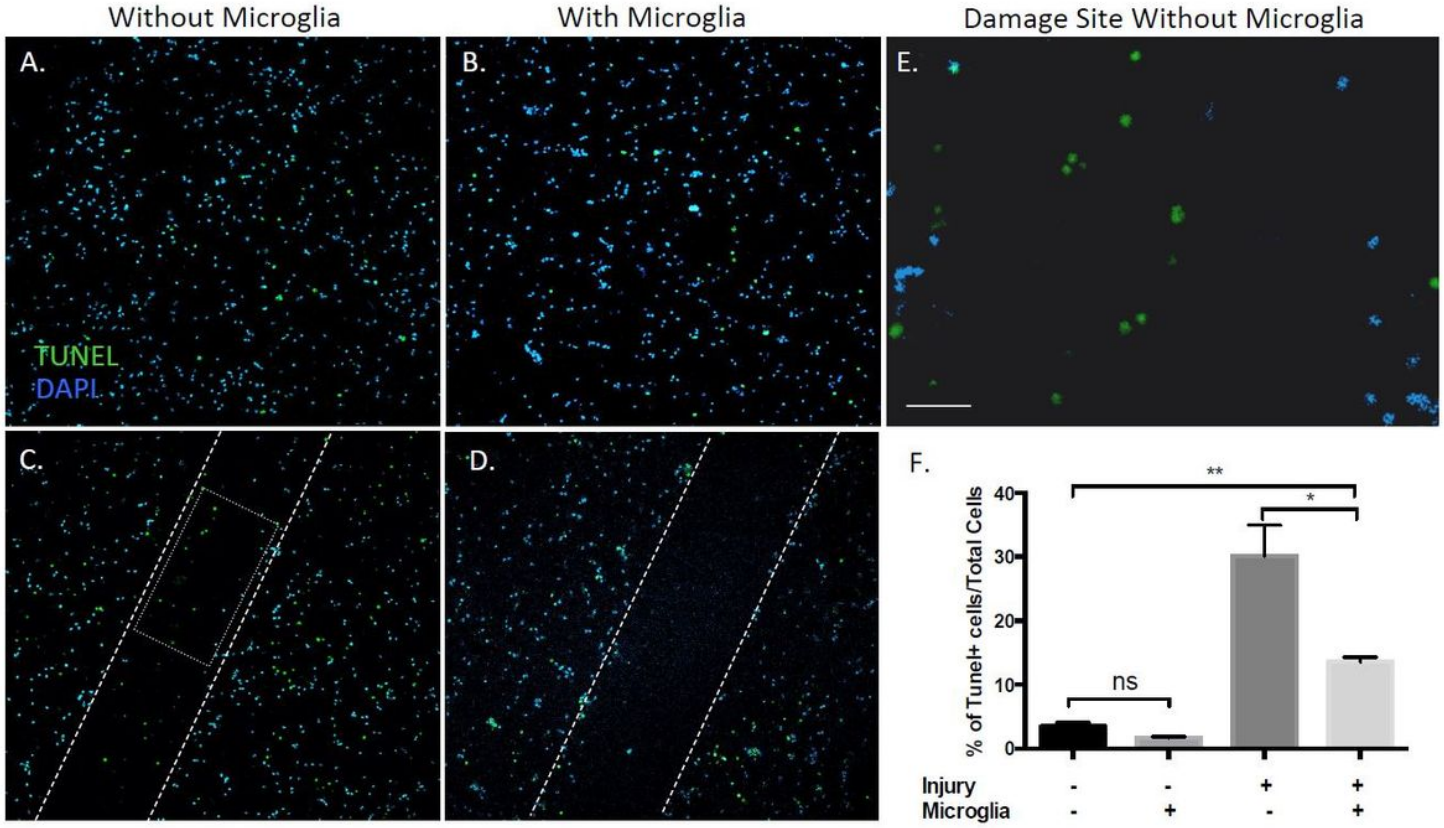


Figure 4

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.

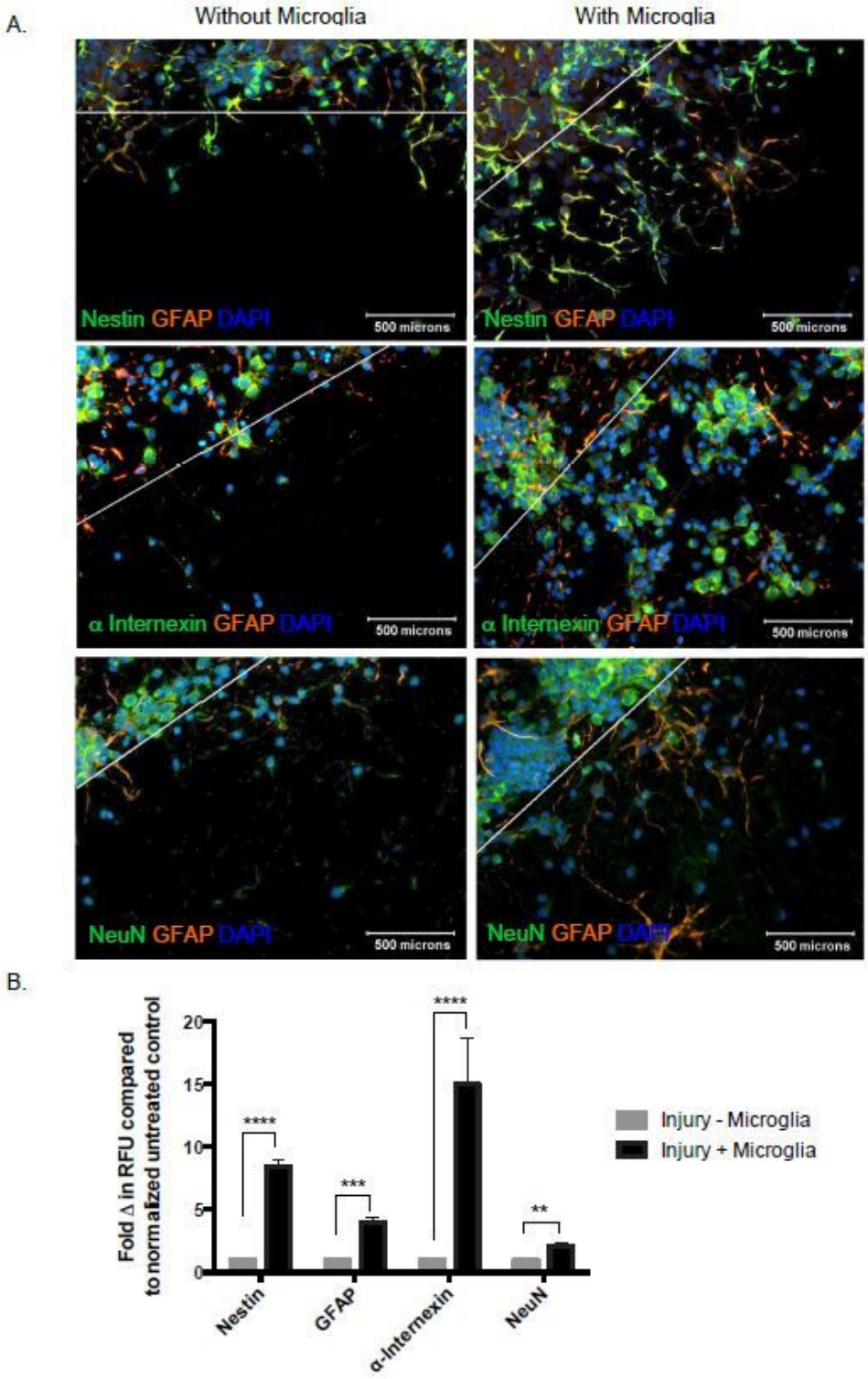


Figure 5

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.

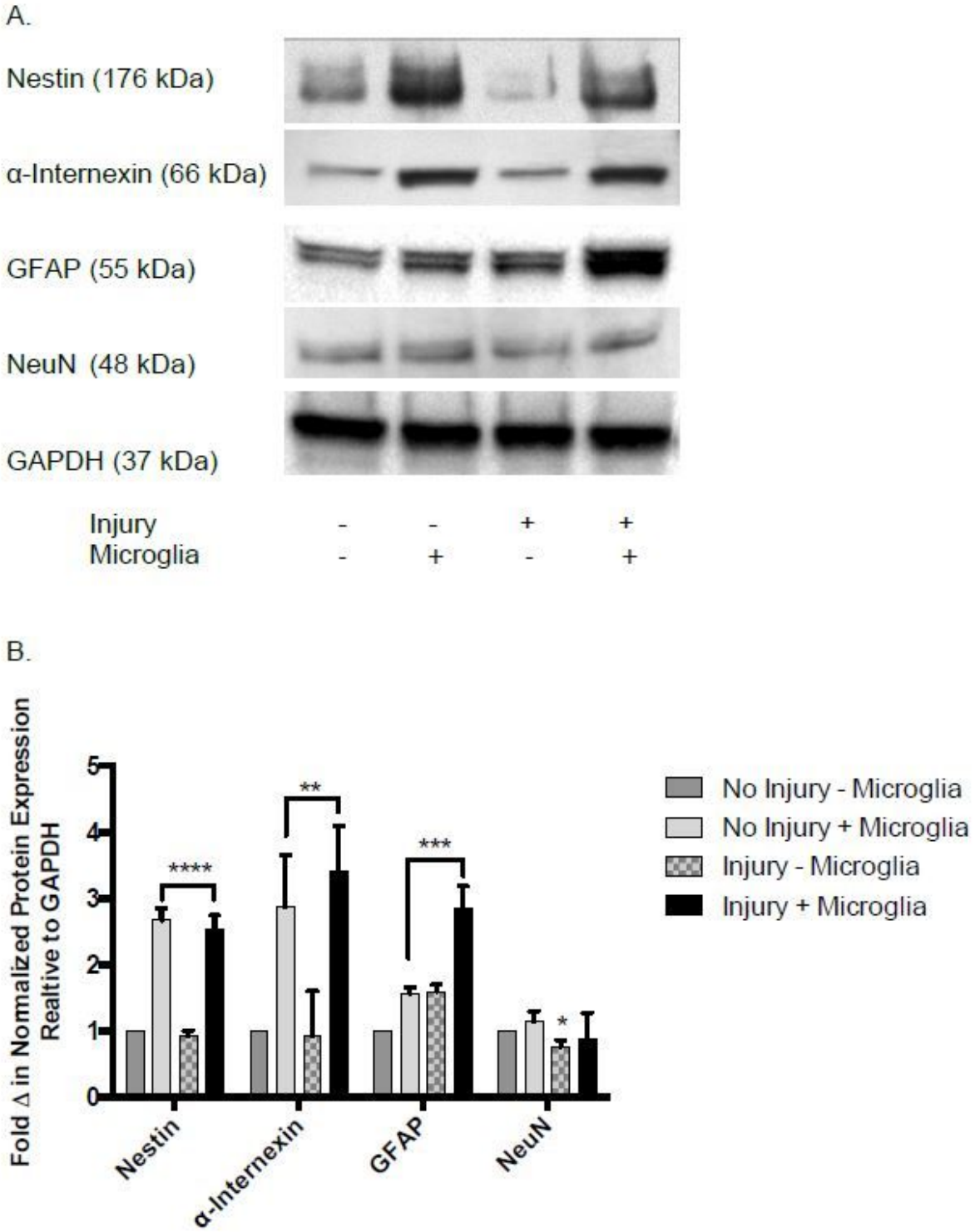


Figure 6

Western blot analysis of Nestin, α-internexin, NeuN, and GFAP expression in uninjured and injured neuronal cultured exposed to 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. One-way ANOVA was used to determine significance. Significance is *$p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$.

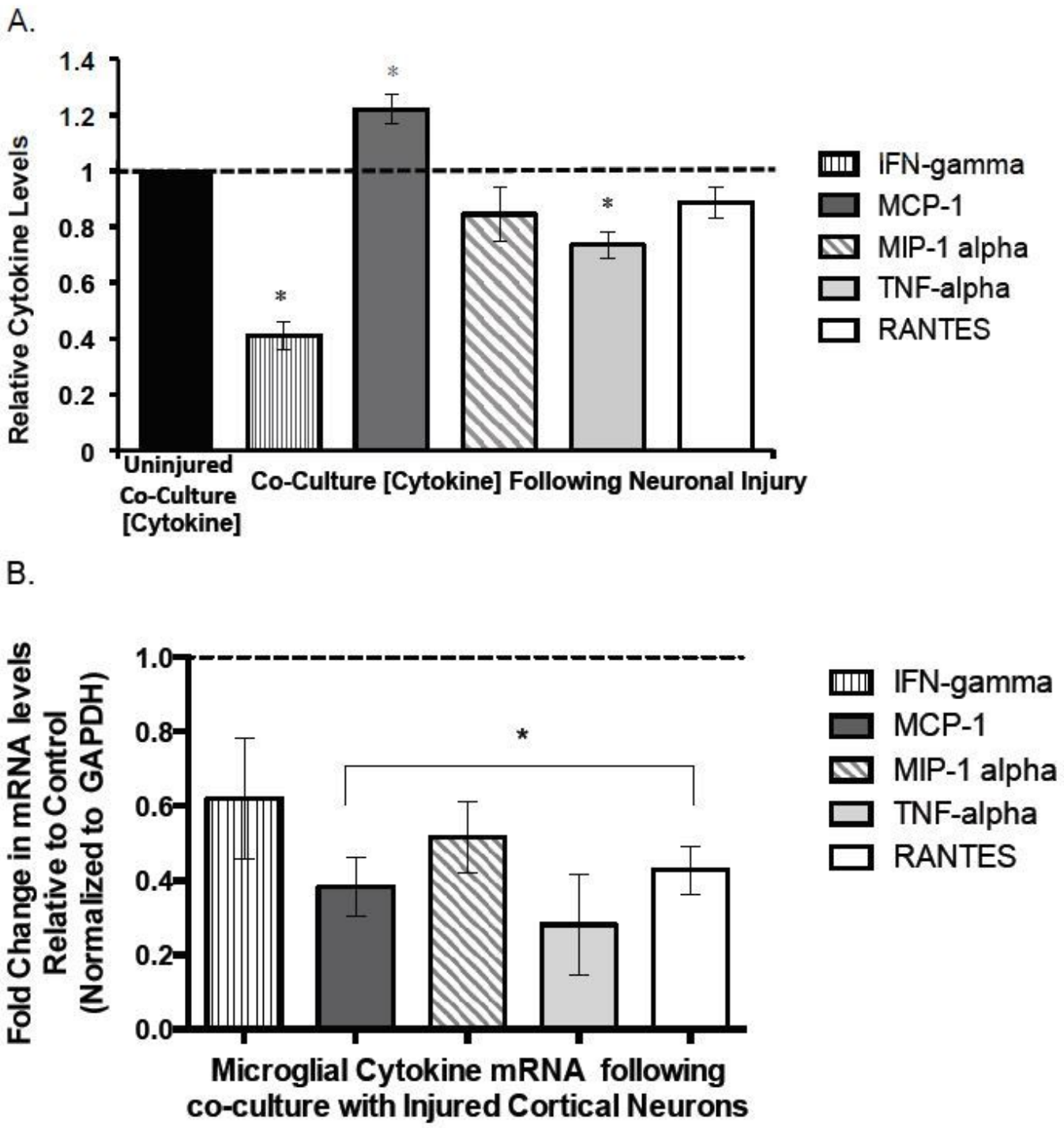


Figure 7

Multiplex ELISA and RT-PCR analyses of inflammatory cytokine protein and mRNA following co-culture with uninjured or injured cortical cells. A) The black bar represents the normalized cytokine levels for IFN-γ, MCP-1, MIP-1α, TNF-α, and RANTES in media collected from co-cultures of microglia and uninjured cortical cells (Uninjured co-culture [cytokine]). The normalized uninjured co-culture cytokine concentrations (Uninjured co-culture [cytokine]) 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 microglial co-culture as compared to the normalized control co-culture cytokine level. Experiments were run in triplicate from three biological replicates. Error bars represent SEM. One-way ANOVA was used to determine significance, *$p<0.05$. B) qRT-PCR analysis of cytokine mRNA levels in microglia following stimulation with injured cortical cells. The fold change in mRNA levels was normalized to Gapdh expression in microglia following stimulation with injured cortical cells is shown. Fold change is compared to mRNA in microglia co-cultured with uninjured neurons (control). 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, *$p<0.05$.

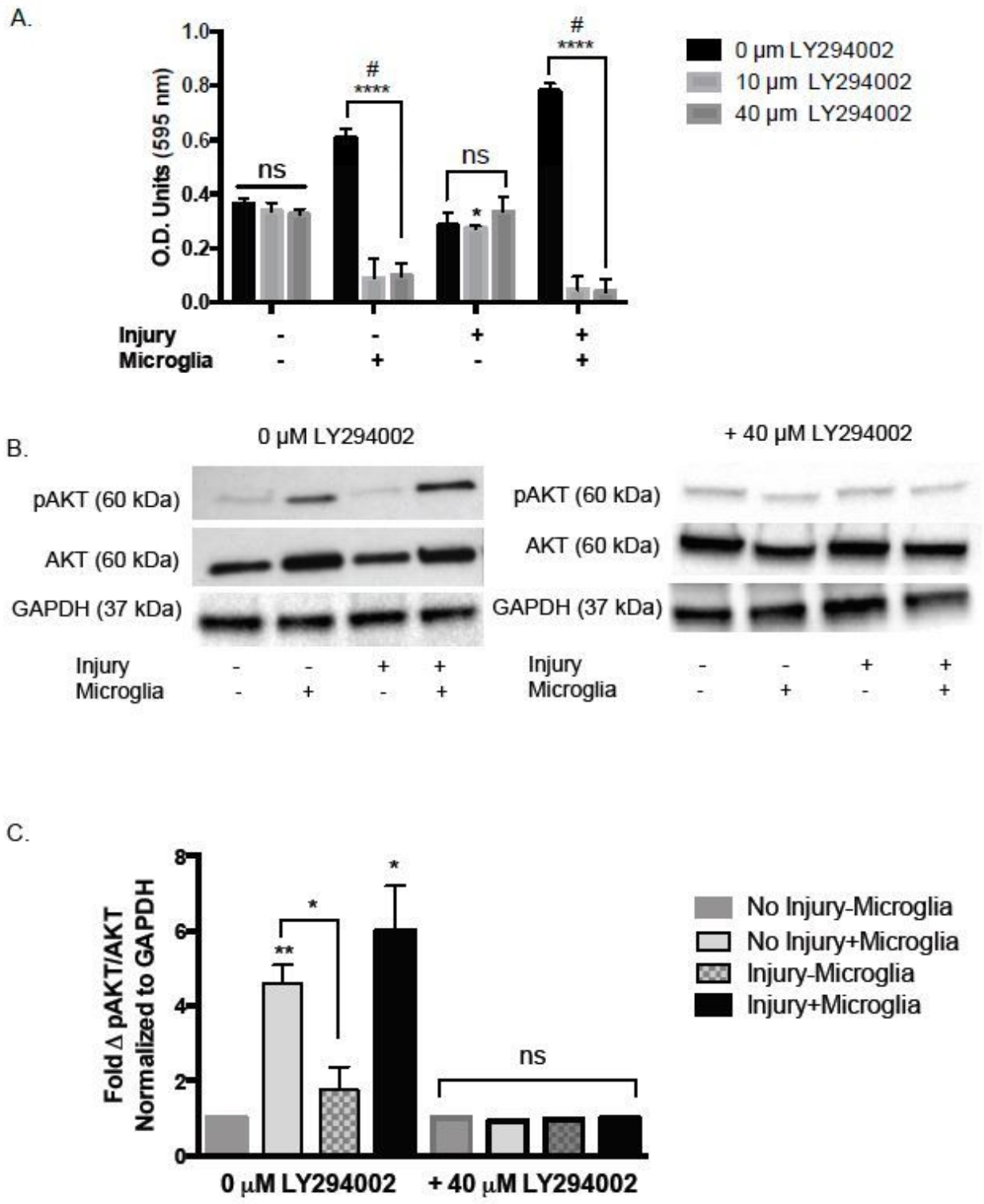


Figure 8

Effect of PI3K inhibition microglial-enhanced cortical cell viability and AKT phosphorylation in cortical cells following 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.

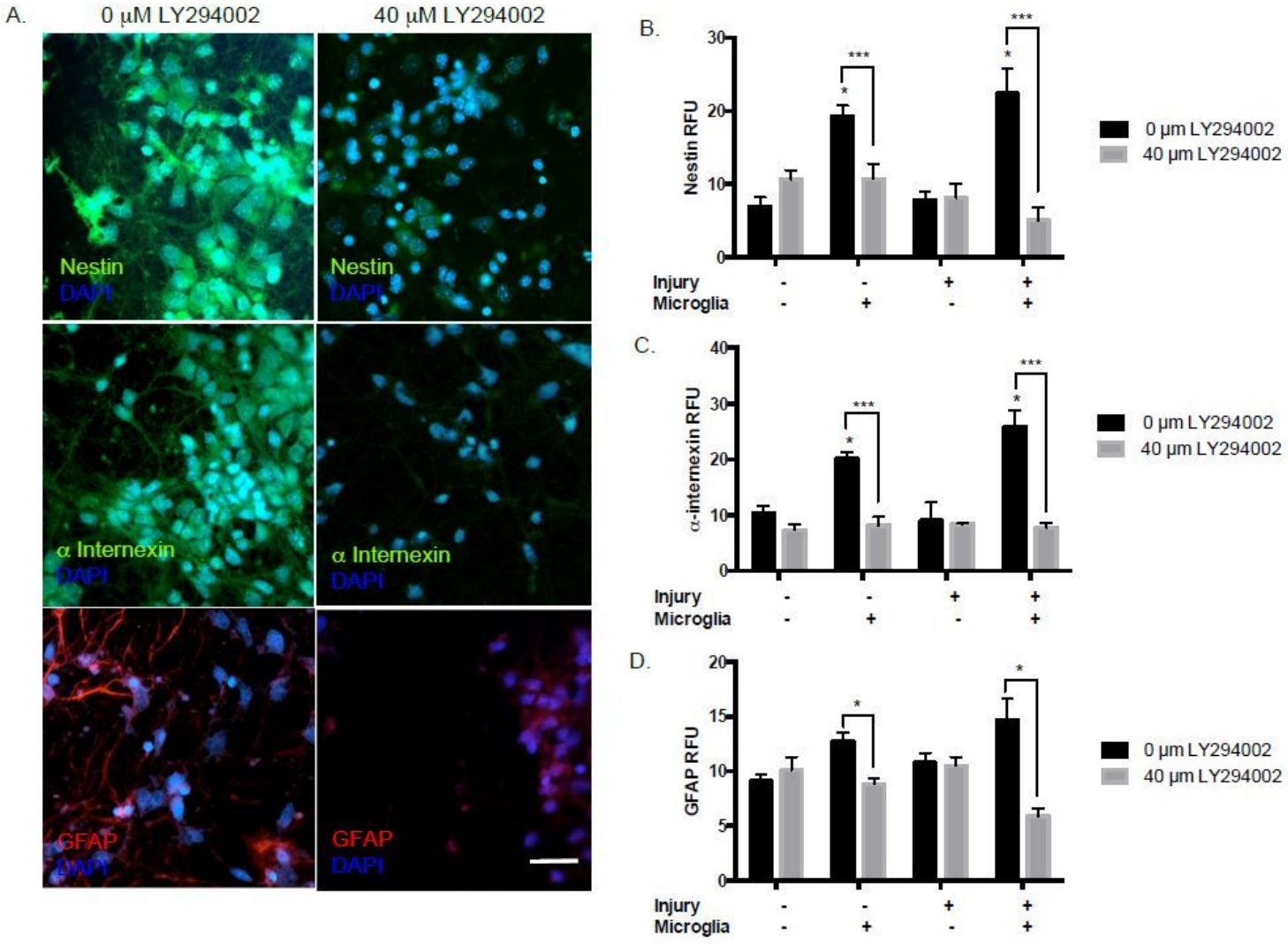


Figure 9

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

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.pdf
- supplement2.pdf
- supplement3.pdf