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Short communication

Myeloid lineage cells inhibit neurite outgrowth through a myosin II-dependent mechanism

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ABSTRACT

The molecular mechanisms that underlie the axonal damage that accompanies CNS inflammation are largely unknown. Here, we investigate the effects of immune cells on neuronal viability and axonal growth and show that conditioned media from myeloid lineage cells inhibit neurite outgrowth without causing apoptosis. Treatment with monocyte conditioned medium enhances myosin light chain phosphorylation in neurons and the neurite outgrowth inhibitory effect of myeloid lineage cells can be attenuated with the myosin II inhibitor blebbistatin. Our results suggest that in the context of CNS inflammation myeloid cells may limit axonal repair in the CNS via a myosin II-dependent mechanism.

1. Introduction

In the CNS, inflammation can occur after acute injury or during the course of disease. Autoimmune diseases such as multiple sclerosis as well as neurodegenerative diseases have important inflammatory components (Amor et al., 2010). In each condition, the degree of axonal damage and the extent of repair contribute to clinical outcome. The contribution of inflammatory cells and their products to axonal injury and repair is not fully understood. Immune cell subsets can produce both pro-inflammatory and anti-inflammatory cytokines in addition to neuroprotective factors including brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor family ligands (Kerschensteiner et al., 1999; Kerschensteiner et al., 2009). Co-culture of immune cells and neurons is one approach to study the effects of individual immune cell subsets on neurons and associated molecular mechanisms of action. Previous studies have demonstrated that activated human CD4+ and CD8+ T cells kill human fetal neurons (Giuliani et al., 2003) and major histocompatibility complex class I/peptide-restricted CD8(+) T lymphocytes can directly lesion hippocampal neurites (Medana et al., 2001).

In contrast, CD4+ T cells promote outgrowth of embryonic cortical neurons (Ishii et al., 2010). Outgrowth from post-natal rat cerebellar neurons is inhibited by activated CD8+ T cells and NK cells and promoted by CD4+ T cells (Pool et al., unpublished observations). These studies demonstrate that immune cells can directly impact on both neuronal viability and axonal integrity but the mechanism of action has not been fully addressed.

We have utilized an *in vitro* system to study the impact of immune cell soluble products on post-natal cerebellar neuron outgrowth and viability. Here we report a sub-lethal inhibitory effect of peripheral blood mononuclear cell (PBMC) conditioned media on neurite outgrowth of cerebellar neurons. We find that the inhibitory activity can be attributed to products from myeloid lineage cells and that the neurite outgrowth inhibitory response is mediated through a myosin II-dependent mechanism. Understanding how the immune system impacts neurons is an important step in developing therapeutic strategies for axonal repair in the context of CNS inflammation.

2. Materials and methods

2.1. Preparation of immune cell conditioned medium

Approval by the Montreal Neurological Institute institutional review board and the animal care committee (according to Canadian Council on Animal Care guidelines) was obtained for all procedures. PBMCs were isolated by Ficoll–Paque density centrifugation from peripheral blood collected by venous phlebotomy from healthy adult volunteers. Cells were cultured in Ultraculture serum-free medium (Cedarlane Laboratories, Burlington, ON) containing 1% penicillin–streptomycin (P/S, Invitrogen, Burlington, ON) and 2 mM glutamine

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(Invitrogen). Monocytes were isolated from PBMCs using CD14 microbeads and MACS technology (Miltenyi Biotec, Auburn, CA). Cell subset purity was greater than 90% as assessed by flow cytometry. Activation was with 100 ng/ml IFNγ (R&D Systems, Minneapolis, MN) and, 2 h later, 100 ng/ml LPS (Invitrogen, Burlington, ON), maintained for 24 h *in vitro*. For *in vitro* differentiated macrophages, CD14+ cells were incubated with 25 ng/ml M-CSF (Peprotech, Rocky Hill, NJ) for 6 days. Half of the media were replenished on day 3. Adult microglia were isolated from normal appearing white matter of temporal lobe brain tissue from patients undergoing surgery for non-tumour related intractable epilepsy (Yong and Antel, 1992). Control conditioned medium was collected from wells lacking immune cells cultured in parallel under the same conditions.

2.2. Outgrowth and viability assays

Total cerebellar neurons from P8 Sprague–Dawley rat pups (Charles River Canada, Montreal, QC) were prepared as described previously (Hsieh et al., 2006) and plated on 96-well plates in DMEM containing 1% N2 supplement (Invitrogen), 1% P/S, 0.08 μg/ml triiodothyronine (Sigma-Aldrich Canada, Oakville, ON), and 0.1 μg/ml L-thyroxine (Sigma-Aldrich Canada). Neurons were cultured for 1 h prior to replacement of the medium with immune cell conditioned medium supplemented with 1% N2 supplement, 0.08 μg/ml triiodothyronine, and 0.1 μg/ml L-thyroxine. 24 h later, neuronal cultures were fixed with 4% paraformaldehyde in PBS and stained with mouse anti-βIII tubulin antibody (Covance, Berkeley, CA) and Hoescht 33342 (Sigma-Aldrich Canada). Neurite outgrowth per cell was determined using the Neurite Outgrowth module of MetaXpress to measure the neurite lengths and the Multi-Wavelength Cell Scoring module of MetaXpress to count the neurons. Apoptosis was assessed using an In Situ Cell Death Detection Kit (Roche Applied Science, Laval, QC) according to the manufacturer's protocol and scored using the Multi-Wavelength Cell Scoring module of MetaXpress.

2.3. Assessment of MLC2 phosphorylation

Cerebellar neurons were plated on PLL-coated glass coverslips. After 24 h in culture, the medium was replaced with pre-warmed supplemented monocyte conditioned medium or control medium for 30 min. Cultures were fixed with 4% paraformaldehyde in PBS pre-warmed to 37 °C for 20 min at room temperature and stained with mouse anti-phosphorylated S19 MLC2 (Cell Signaling Tech) and rabbit anti-βIII tubulin (Covance). For quantification, mean pixel intensities were measured in ImageJ and the fold-change was calculated as a ratio of the value obtained in neurons exposed to monocyte sups to that obtained in neurons exposed to control medium. For some experiments, neuronal cultures were loaded with 2.5 μM CellTracker Green CMFDA (Invitrogen) for 30 min prior to exposure to monocyte conditioned medium.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The specific tests used, the *p*-values obtained and the number of independent experiments analyzed are reported in the figure legends and text. For ANOVA analyses, post-tests were performed and the significant comparisons are indicated by * ($p<0.05$), ** ($p<0.01$), or *** ($p<0.001$). Data were considered statistically significant if $p<0.05$.

3. Results

3.1. Immune cell soluble products inhibit neurite outgrowth

Soluble products from immune cells were evaluated for their ability to affect cerebellar neuron outgrowth. Cerebellar neurons from P8 rats were used because they have matured to a stage in which they respond to outgrowth inhibitory cues, a characteristic of adult neurons (Cai et al., 2001; Piper et al., 2007; Hannila and Filbin, 2008). A robust dose-dependent decrease in neurite outgrowth is observed when cerebellar neurons are cultured in the presence of PBMC conditioned medium (Fig. 1). Because previous reports have indicated that immune cells can kill embryonic neurons (Giuliani et al., 2003), we assessed cell death in our cultures using a TUNEL assay for DNA damage. The number of TUNEL positive neurons is not affected by PBMC conditioned medium thus the immune cell-dependent neurite outgrowth inhibition is not a by-product of neuronal death but a sub-lethal effect. We also see no evidence of neuritic beading in response to microglia, a phenomenon that has been reported in embryonic cortical neurons (Takeuchi et al., 2005) suggesting that the responses of mature neurons are distinct.

3.2. Monocyte soluble products inhibit neurite outgrowth

PBMCs contain a mixture of circulating immune cell subsets. To determine the cell types mediating the inhibitory effect, supernatants from immune cell subsets were assessed for their ability to inhibit neurite outgrowth. We find that monocyte supernatants significantly inhibit neurite outgrowth (Fig. 2) whereas the supernatants of other cell subsets (CD3+ T cells, B cells, and NK cells) have no effect even upon activation (data not shown).

3.3. Other myeloid lineage cells also produce neurite outgrowth inhibitory factors

Cells of the myeloid lineage that could be in close proximity to axons *in vivo* include macrophages and microglia. We therefore assessed the effects on neurite outgrowth of supernatants from monocytes differentiated into macrophages *in vitro* and primary human adult microglia. The soluble products of both of these cell types exert inhibitory effects similar to those of monocyte supernatants (Fig. 2).

3.4. The outgrowth inhibitory effect is myosin II dependent

To address the mechanism underlying reduced neuronal growth in the presence of immune cells, we screened several pharmacological inhibitors of known modulators of neurite outgrowth or degeneration for their ability to reverse the inhibitory effect of the supernatants (Table 1). Blebbistatin significantly attenuates the inhibitory effect of all myeloid lineage cell supernatants (Fig. 3A), while the other inhibitors have no effect (data not shown). These results suggest that secreted factors from myeloid lineage cells signal through myosin II to inhibit neurite outgrowth. To test this, we examined the levels of active phosphorylated myosin light chain (p-MLC2) in neurons exposed to stimulated monocyte supernatants. We observe an increase in p-MLC2 in growth cones in response to stimulation (Fig. 3B). p-MLC2 levels increase $1.68+/-0.14$ fold in the growth cone, a statistically significant change ($p=0.0419$ by one-sample *t*-test compared to a hypothetical value of 1; 3 experiments of at least 20 neurons per condition). No significant change in p-MLC2 signal is detected in the cell body or neurite shaft. A similar increase in p-MLC2 was observed in two experiments in which the p-MLC2 signal was normalized to a CellTracker Green signal to control for volumetric effects ($2.15+/-0.37$ fold increase).

4. Discussion

We report that neurite outgrowth can be inhibited by soluble factors from human myeloid lineage cells including activated monocytes, *in vitro* differentiated macrophages, and adult primary microglia. This inhibitory effect is dependent on neuronal myosin II activity. Supernatants from cells of the myeloid lineage activate myosin in

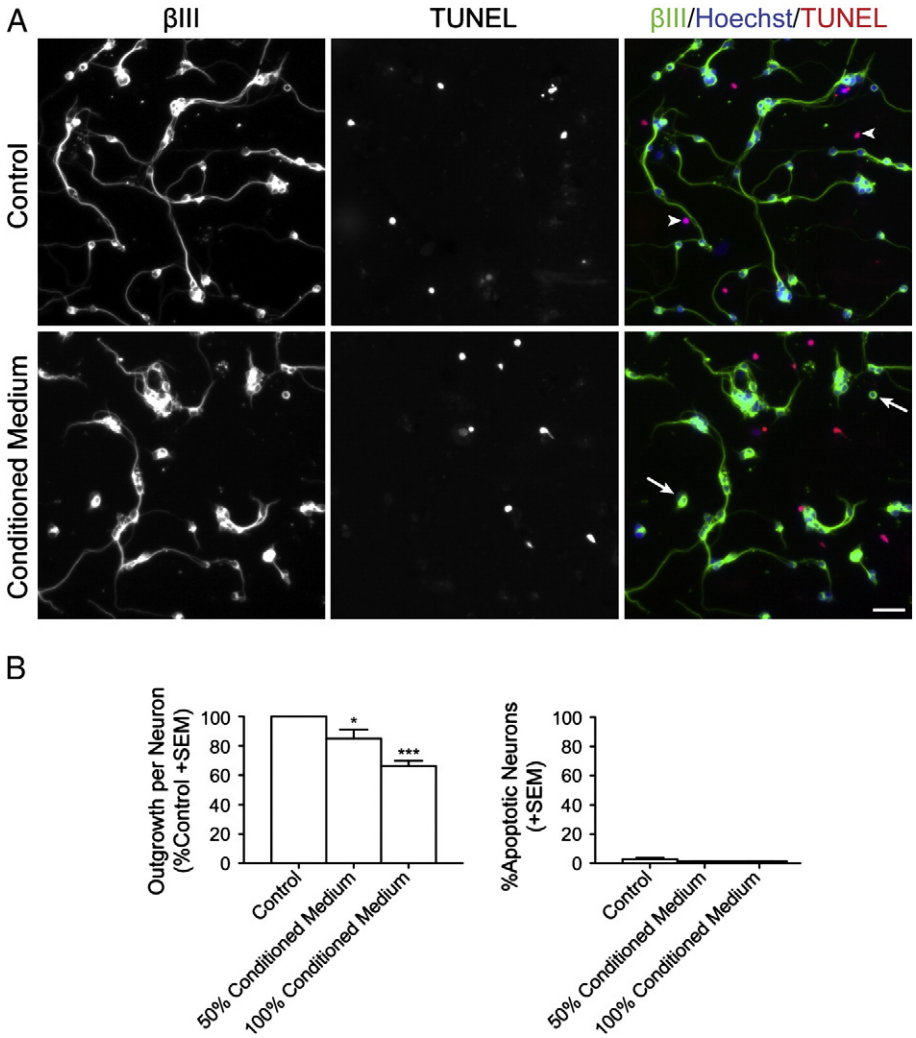

Fig. 1. PBMCs inhibit neurite outgrowth without enhancing apoptosis. A) Representative images of untreated neurons (Control) and neurons cultured with conditioned medium from human PBMCs. Neurons are triple stained with βIII-tubulin antibody, Hoescht nuclear stain and TUNEL. Note that many neurons without neurite outgrowth remain TUNEL negative (arrows). Some βIII-tubulin negative (non-neuronal) cells are TUNEL positive (arrowheads). Scale bar = 50 μm. B) Quantification of human immune cell effects on neurite outgrowth/cell (n ≥ 5) and apoptosis (n = 3). Statistical analysis by one-way repeated measures ANOVA indicates a significant effect of PBMC supernatants on neurite outgrowth ($p < 0.0001$), $^{*}p < 0.05$, $^{***}p < 0.001$ compared to control by Tukey's post-tests but no significant effect on apoptosis ($p = 0.1116$).

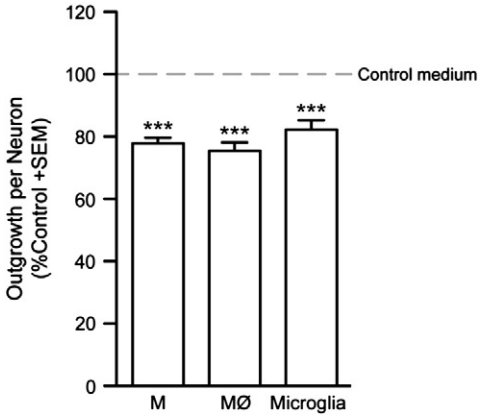

Fig. 2. Soluble products of myeloid lineage cells inhibit neurite outgrowth. Quantification of neurite outgrowth from cerebellar neurons cultured in the presence of supernatants from immune cells expressed as a percentage of the control medium collected from parallel wells cultured without immune cells (n ≥ 5). M = monocytes, MØ = macrophages. Statistical analysis by one-sample *t*-test compared to a hypothetical value of 100% indicates a significant effect of stimulated monocytes ($p = 0.0002$, n = 5), *in vitro* differentiated macrophages ($p = 0.0008$, n = 5), and microglia ($p = 0.0003$, n = 9).

neurons through phosphorylation of its regulatory light chain. Very little is known about how lost axonal connections may be repaired but it is clear that clinical outcome will depend on the degree of damage inflicted and the mechanisms in place to repair that damage. Our data suggest that persistence of macrophages and presence of microglia at CNS lesion sites could actively inhibit regrowth of axons through expression of soluble inhibitory factors.

The results of our study are consistent with a previous study demonstrating that conditioned media from LPS-stimulated BV-2 cells can inhibit outgrowth from the motor neuron cell line, NSC-34 (Li et al., 2007); however our results are surprising when considering *in vivo* data demonstrating that zymosan- or lens injury-induced macrophage recruitment into the vitreous fluid of the eye promotes retinal ganglion cell regeneration after optic nerve crush (Leon et al., 2000; Yin et al., 2003). Another study suggests that zymosan stimulation of macrophages can exert both beneficial and deleterious effects on dorsal root ganglion neurons depending on the degree of exposure of the axons to the activated macrophages (Gensel et al., 2009). Together with our data, a reasonable interpretation is that myeloid lineage cells may promote or inhibit neuronal growth depending on the experimental paradigm and the activation state of the cells. Distinct modes of activation will dictate the repertoire of beneficial and destructive molecules expressed by

Table 1
Pharmacological inhibitors used in neurite outgrowth assays.

Inhibitor	Target	Source	Concentration
Y-27632	Rho-kinase	EMD Biosciences	10 μM
PI-103	PI3kinase	EMD Biosciences	500 nM
Wortmannin	PI3kinase	EMD Biosciences	100 nM
Akti1/2	Akt1/2	EMD Biosciences	1 μM
6-bromoindirubin-3'-acetoxime	GSK3	EMD Biosciences	300 nM
CT99021	GSK3	Gift from C. Sutherland	2 μM
blebbistatin	Myosin II	Sigma-Aldrich Canada	50 μM
purvalanol A	Cdk1, Cdk2, Cdk5	EMD Biosciences	10 μM
U0126	MEK	Sigma-Aldrich Canada	10 μM
PD098059	MEK	Sigma-Aldrich Canada	10 μM
Gö6976	PKC	Sigma-Aldrich Canada	100 nM
z-DEVD-fmk	Caspase-3	BD Biosciences	10 μM
z-VEID-fmk	Caspase-6	EMD Biosciences	10 μM

immune cell subsets and subsequent outcomes in CNS inflammation (Kerschensteiner et al., 2009). Identification of myeloid-derived inhibitors may suggest a therapeutic strategy to optimize favorable immune cell effects while neutralizing inhibitory properties.

4.1. Mechanism of action of myeloid lineage cell neurite outgrowth inhibition

We find that myeloid lineage cell mediated inhibition of neurite outgrowth is myosin II dependent as it is reversed in the presence of blebbistatin, but, surprisingly, Rho-kinase-independent as Y-27632 fails to modify the neurite outgrowth inhibition phenotype. These results suggest that immune cell soluble products can activate myosin II in neurons leading to neurite outgrowth inhibition that is independent of Rho-kinase activation. This is interesting, as myosin II activation often requires Rho-kinase activity (Amano et al., 1996; Amano et al., 1998). However several other kinases, including integrin-linked kinase (Deng et al., 2001; Kiss et al., 2002; Muranyi et al., 2002), PAK (Takizawa et al., 2002), ZIP kinase (Murata-Hori et al., 1999), ZIP-like kinase (MacDonald et al., 2001), and myotonic dystrophy protein kinase (Muranyi et al., 2001), can regulate the two major upstream regulators of myosin II activity, myosin light chain kinase (active upon phosphorylation) and myosin phosphatase (inactive upon phosphorylation), to activate myosin II. It is possible that one of these kinases contributes to myosin II activation during myeloid lineage cell induced neurite outgrowth inhibition.

While we have identified myosin II as a downstream target of the myeloid lineage cell inhibitory effect in neurons, the soluble factors

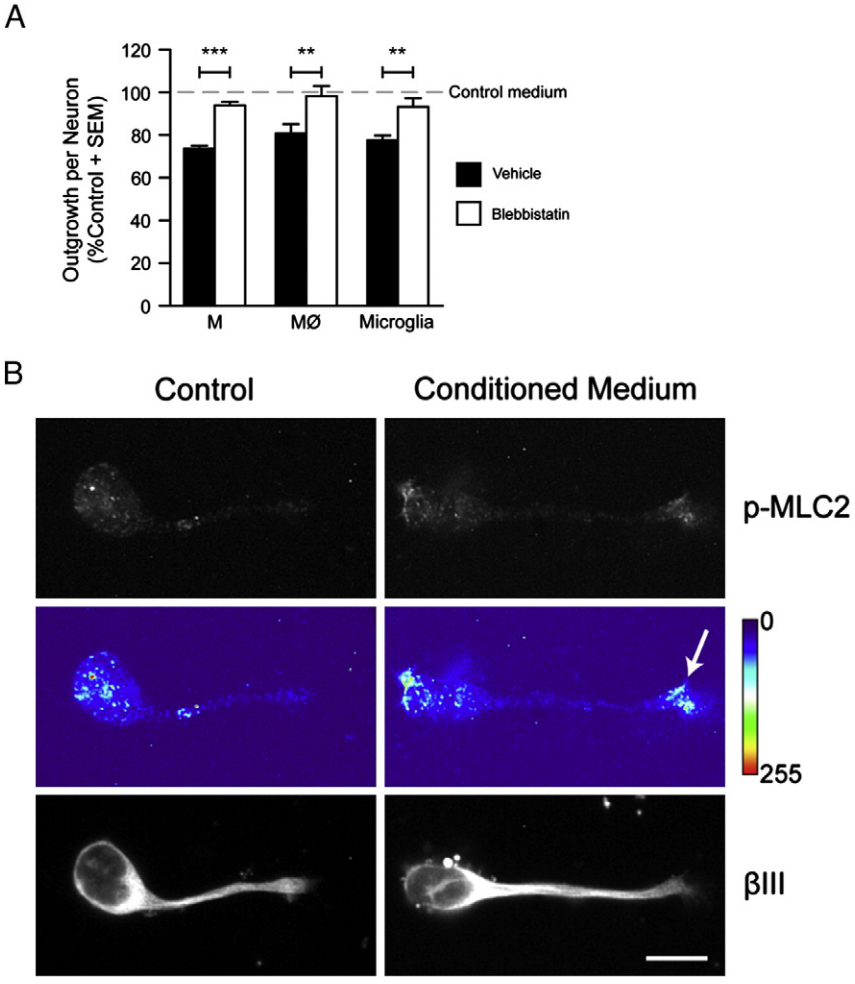

Fig. 3. Myosin II activity is required for inhibition of cerebellar neurite outgrowth by myeloid lineage cell conditioned medium. A) Quantification of neurite outgrowth from cerebellar neurons cultured in the presence of myeloid lineage cell conditioned medium with blebbistatin or vehicle (DMSO) expressed as a percentage of the outgrowth of neurons grown in the presence of blebbistatin or DMSO alone ($n \geq 4$). Statistical analysis by two-way repeated measures ANOVA indicates significant enhancement of neurite outgrowth by blebbistatin ($p<0.0001$), $^{**}p<0.01$, $^{***}p<0.001$ compared to vehicle control by Bonferroni post-tests. B) Representative images showing neurons stained for MLC2 phosphorylated at S19 (top; p-MLC2), a heat map of the p-MLC2 signal (middle), and βIII-tubulin (bottom; βIII) in the presence of control (left; control) or monocyte conditioned medium (right). Scale bar = 10 μm. The arrow highlights the strong staining in the growth cone of the neuron exposed to monocyte conditioned medium.

responsible for the inhibitory phenotype remain to be identified. Several of the pro-inflammatory molecules secreted by myeloid lineage cells have been shown to impact neuronal health (Allan and Rothwell, 2001). In addition to the classical secreted immune molecules, macrophages and microglia can express several molecules originally identified as inhibitors of neuronal growth including semaphorins, repulsive guidance molecule, slits and netrins (Schwab et al., 2005; Wehrle et al., 2005; Ji et al., 2009; Nkyimbeng-Takwi and Chapoval, 2011). Further studies into the molecular mechanism of myeloid cell mediated neurite outgrowth inhibition will require attention to the possibility that more than one pathway is involved and identification of the signals that regulate expression of the soluble inhibitory factors.

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