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Neurite outgrowth is differentially impacted by distinct immune cell subsets

Madeline Pool [a], Isabel Rambaldi [a], Peter J. Darlington [a,b], Melissa C. Wright [a,b], Alyson E. Fournier [a,*], Amit Bar-Or [a,b,**]

[a] Department of Neurology and Neurosurgery, Montreal Neurological Institute, 3801 Rue University, Montreal, Quebec, Canada, H3A 2B4
[b] Department of Neurology and Neuroimmunology Unit, Montreal Neurological Institute, 3801 Rue University, Montreal, Quebec, Canada, H3A 2B4

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ABSTRACT

Axonal damage can occur in the central nervous system following trauma, during the course of autoimmune and neurodegenerative disease and during viral and bacterial infections. The degree of axonal damage and absence of spontaneous repair are major determinants of long-term clinical outcome. While inflammation is a common feature of these conditions, the impact of particular immune cell subsets and their products on injured axons is not fully known. To investigate the impact of immune cells on neuronal viability and axonal repair, we developed an in vitro culture system in which neurons are exposed to mixed or distinct immune cell subsets. We find that total peripheral blood mononuclear cells (PBMCs) have a significant inhibitory effect on neurite outgrowth that is independent of apoptosis. Using isolated immune cells subsets, we demonstrate that activated CD4+ T cells enhance neurite outgrowth while activated NK cells and CD8+ T cells inhibit neurite outgrowth. We find that NK cell inhibition of neuronal outgrowth is dependent on MAPK activity. Our findings describe heterogeneous effects of individual immune cell subsets on neuronal growth and offer important insights into the cellular and molecular mechanisms that may impact axonal repair in inflammatory CNS conditions.

Introduction

The degree of axonal damage in the central nervous system following trauma or in the course of disease is a major determinant of long-term clinical outcome. This is certainly true following acute injuries such as spinal cord injury and is now appreciated to be a major cause of persistent neurological disability in other conditions such as multiple sclerosis (MS) (De Stefano et al., 1998) where clinical disability scores increase with increasing levels of axonal loss (Wujek et al., 2002). The microenvironment surrounding a lesioned axon plays a critical role in aiding or inhibiting axon repair. Inflammatory cells and their products represent important components of the local environment in the context of such injuries. CNS inflammation is well documented after acute injury such as spinal cord trauma, in pathogenic diseases including parasitic, viral and bacterial infections and in autoimmune diseases like MS. It has also more recently been recognized as a component of neurodegenerative diseases including Alzheimer's (AD), Parkinson's (PD) and Amyotrophic Lateral Sclerosis (ALS) (Amor et al., 2010).

The relationship between inflammation and axon repair following damage is complex; inflammation can have both detrimental and beneficial effects (Popovich and Longbrake, 2008; Schwartz et al., 2009). In spinal cord injury models, functional improvements can be detected by restricting the recruitment of blood-derived leukocytes while T and B cells seem to exhibit neurotoxic properties as well as reparative activity (Popovich and Longbrake, 2008). Many therapies directed at limiting the acute inflammatory response are being pursued as a strategy to improve recovery following spinal cord injury yet microglia and macrophages play critical roles in removing cellular debris and overcoming the impact of axon outgrowth inhibitors (Alexander and Popovich, 2009). Further, macrophage activation through zymosan injection can promote retinal ganglion cell regeneration (Leon et al., 2000; Yin et al., 2003) and can render the spinal cord environment permissive for axon growth (Gensel et al., 2009). Understanding the impact of individual immune cell subsets and their products on axon viability and repair is thus an important issue for optimizing immunomodulatory therapies to improve axon regeneration.

The effects of immune cells on healthy or compromised axons are not well understood, in part because primary adult human CNS neurons are not available for study. Activated human CD4+ and CD8+ T cells kill human fetal neurons through a contact-dependent mechanism in a 24-hour co-culture paradigm (Giuliani et al., 2003). CD8+ antigen/MHC-restricted T cells mediate axon transection of

* Correspondence to: A. Fournier, Montreal Neurological Institute, 3801 Rue University, BT-109, Montreal, PQ, Canada, H3A 2B4. Fax: +1 514 398 6547.
** Correspondence to: A. Bar-Or, Montreal Neurological Institute, 3801 Rue University, Montreal, PQ, Canada, H3A 2B4.
E-mail addresses: alyson.fournier@mcgill.ca (A.E. Fournier), amit.bar-or@mcgill.ca (A. Bar-Or).

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MHC I-expressing E16 hippocampal neurons (Medana et al., 2001). In contrast, Th1 cells promote outgrowth when co-cultured with embryonic cortical neurons (Ishii et al., 2010). These studies demonstrate that immune cells directly impact neuronal viability and axonal integrity both positively and negatively.

In this study we systematically investigate the effects of peripheral blood mononuclear cells (PBMCs) and their subsets on neurite outgrowth in an in vitro co-culture assay. We use immune cells from adult human donors as our eventual goal is to understand how human immune cell subsets may mediate effects that could eventually be targeted in human disease. As primary mature human neurons remain unavailable, we chose post-natal rat cerebellar neurons for the target population since these are from a mature developmental stage when neurons are sensitive to axon growth inhibitors (Cai et al., 2001; Hannila and Filbin, 2008; Piper et al., 2007). We find that activated PBMCs inhibit neurite outgrowth of cerebellar neurons. Our analysis of immune cell subsets indicates that NK cells and CD8+ lymphocytes contribute to the neurite outgrowth inhibitory effect of the PBMC population. In contrast, CD4+ T cells significantly promote neurite extension. Inhibition of p42/p44 MAPK attenuates the inhibitory activity of NK cells indicating that the MAPK pathway may be therapeutically targeted to optimize the beneficial effects of neuroinflammation on axon repair.

Results

PBMCs inhibit neurite outgrowth

To investigate the effects of immune cells on neurite outgrowth we co-cultured either human or rat PBMCs with rat cerebellar neurons. PBMCs were isolated and maintained in culture for 3 days in the presence or absence of PMA/Ionomycin to activate the immune cells. We find that activated human PBMCs significantly inhibit rat cerebellar neurite outgrowth by 47 +/− 4.5% compared to control outgrowth levels and decrease the proportion of neuron bearing neurites while the inactivated PBMCs have no effect (Fig. 1A and Supplementary Fig. 1). In addition, we tested whether other neuronal types were also susceptible to outgrowth inhibition by immune cells. Both embryonic cortical and hippocampal neurons showed decreased outgrowth in the presence of PBMCs (Figs. 1B and C). We confirmed that activated rat PBMCs exhibit a similar neurite outgrowth inhibitory effect, indicating that the observed inhibitory activity is not merely

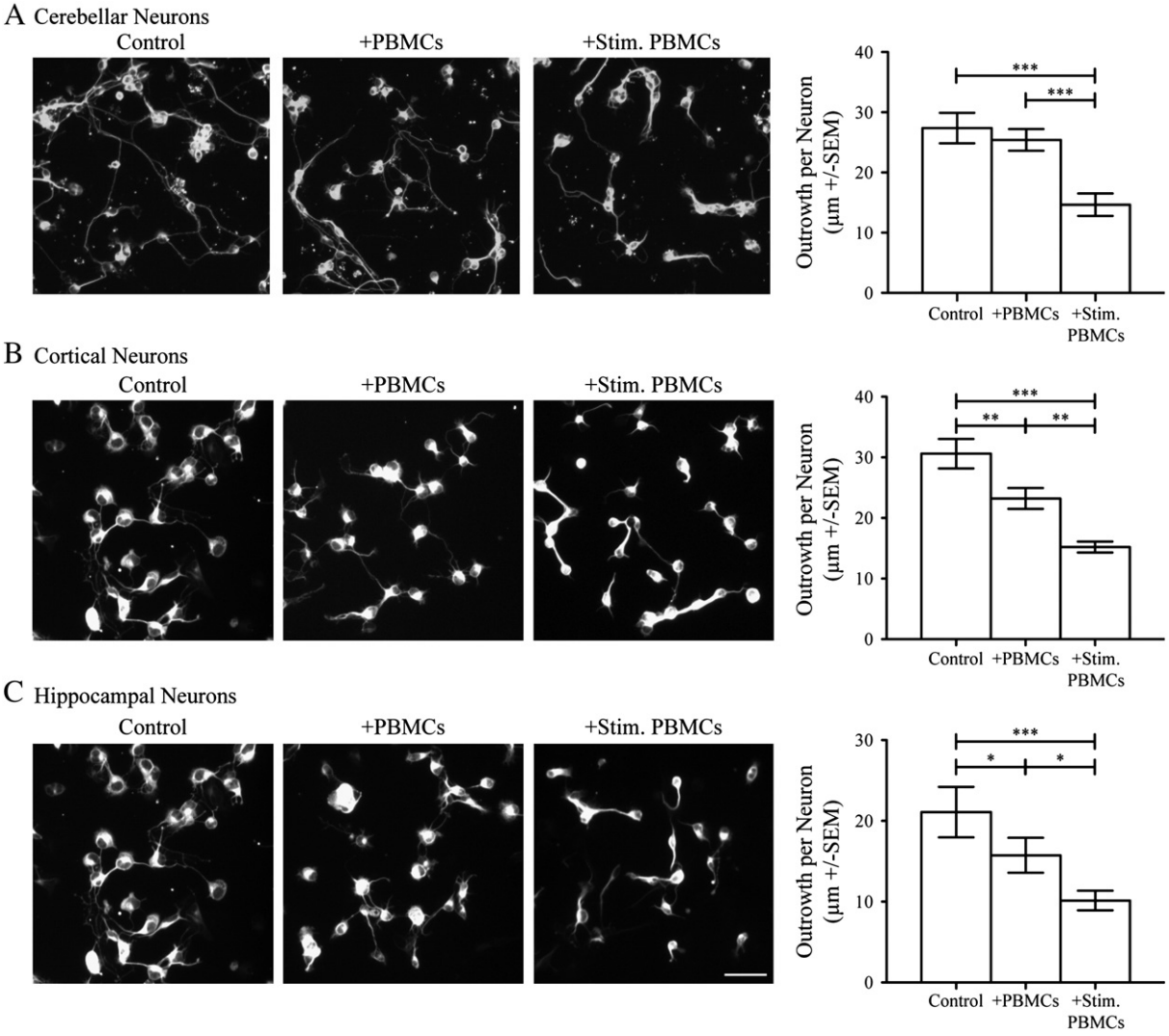

Fig. 1. PBMCs inhibit neurite outgrowth of cerebellar neurons. Representative images of cerebellar (A), cortical (B) and hippocampal (C) neurons either untreated (Control) or co-cultured with unstimulated (+PBMCs) or PMA/ionomycin stimulated PBMCs (+Stim. PBMCs) at a ratio of 2:1 immune cells to neurons. Neurons are stained with βIII tubulin antibody. Scale bar = 50 µm. Graphs represent quantification of human PBMC effects on neurite outgrowth/cell (n = 13 for cerebellar neurons, n = 6 for cortical and hippocampal neurons). Statistical analysis by one-way repeated measures ANOVA indicates significant neurite growth inhibitory effects ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***) by Tukey's post-tests.

an artifact of a xenogeneic system (Supplementary Fig. 2) enabling us to pursue subsequent experiments using human derived immune cells.

Immune cell inhibition of neurite extension is not associated with induction of neuronal apoptosis

Previous reports have indicated that immune cells kill embryonic neurons (Giuliani et al., 2003). To determine if activated PBMCs promote P8 cerebellar neuron cell death, we performed a TUNEL assay to assess DNA damage. Isolated cerebellar neurons in culture display minimal DNA damage and this is not affected by the addition of activated PBMCs (Fig. 2; Table 1). A small proportion of βIII tubulin-negative non-neuronal cells is TUNEL positive in our cultures and this number is also not significantly affected by the addition of activated PBMCs (Fig. 2; data not shown). Thus, the decreased outgrowth mediated by immune cells in our cultures does not merely reflect immune cell mediated killing of neurons, but rather a sub-lethal neurite outgrowth inhibitory effect.

Immune cell subsets have distinct impacts on neurite outgrowth

To assess the influence of different immune cell types on neurite outgrowth, we performed outgrowth assays with purified subsets of the PBMC mixture, either unstimulated, or activated using subset-specific paradigms as indicated in the materials and methods. Unstimulated B cells, T cells, and NK cells do not have a significant impact on neurite outgrowth (Fig. 3). Activation of the subsets however induces subset-specific effects on neurite outgrowth. Activated total CD3+ T cells modestly but significantly inhibit neurite outgrowth (Fig. 3). Notably, the inhibitory property of CD3+ T cells varies widely from donor to donor. Similar variability was observed using T cells derived from rat PBMCs (data not shown). To attempt to address this variability we further divided the human T cell population into CD4+ and CD8+ T cells. Activated CD4+ T cells significantly promote outgrowth whereas activated CD8+ T cells significantly inhibit neurite outgrowth; however, there is still significant donor-to-donor variability in the inhibitory properties of CD8+ T cells. A more robust and highly consistent neurite outgrowth inhibitory effect is observed with NK cells, which decrease outgrowth by 23 +/− 2.8%. This effect is also not accompanied by enhanced neuronal apoptosis; the percentage of TUNEL positive neurons in the cultures is 0.894 +/− 0.108 and 1.03 +/− 0.128% in cultures without and with activated NK cells, respectively.

Table 1
Quantification of apoptotic neurons in co-cultures of cerebellar neurons with PBMCs. DNAse-treated cells were used to confirm kit activity (Nuclease). Statistical analysis by two-way ANOVA indicates no significant change in the percentage of TUNEL positive neurons in the presence of stimulated PBMCs compared to control.

	Control			+Stim. PBMCs		
Time	%TUNEL+ve neurons	SEM	n	%TUNEL+ve neurons	SEM	n
6 h	1.99	0.197	3	0.55	0.094	3
12 h	1.10	0.070	3	0.44	0.074	3
18 h	1.34	0.055	3	1.36	0.249	3
24 h	2.32	0.216	6	2.98	0.560	6
Nuclease (24 h)	99.2	0.346	3			

We next assessed if the neurite outgrowth inhibitory effect of the immune cells occurs through contact-dependent or -independent mechanism by testing the effects of immune cell conditioned media on neurite outgrowth. Conditioned media from B cells, T cells or NK cells fail to alter cerebellar neurite outgrowth (Fig. 4). The lack of effect from the conditioned media is unlikely to be explained by a failure to concentrate a candidate inhibitory cue because conditioned media from cells of the myeloid lineage does significantly inhibit P8 rat cerebellar neurite outgrowth (Pool et al., 2011). To account for the possible effects of short-lived inhibitory cues, we also performed experiments using transwells but observed no significant outgrowth inhibitory effects of either T cells or NK cells (Fig. 4B). In addition, using time-lapse video microscopy, we show that contacts are made between immune cells and neurons in co-culture and that these contact events can have an immediate impact on the growth of the neurites (Supplementary Videos 1 and 2 and Supplementary Fig. 3). Together, these data are consistent with a contact-dependent model of T cell- and NK cell-dependent neurite outgrowth modulation.

NK cells inhibit neurite outgrowth through a MAPK-dependent mechanism

To address the mechanism underlying reduced neurite outgrowth in the presence of NK cells, we screened several pharmacological

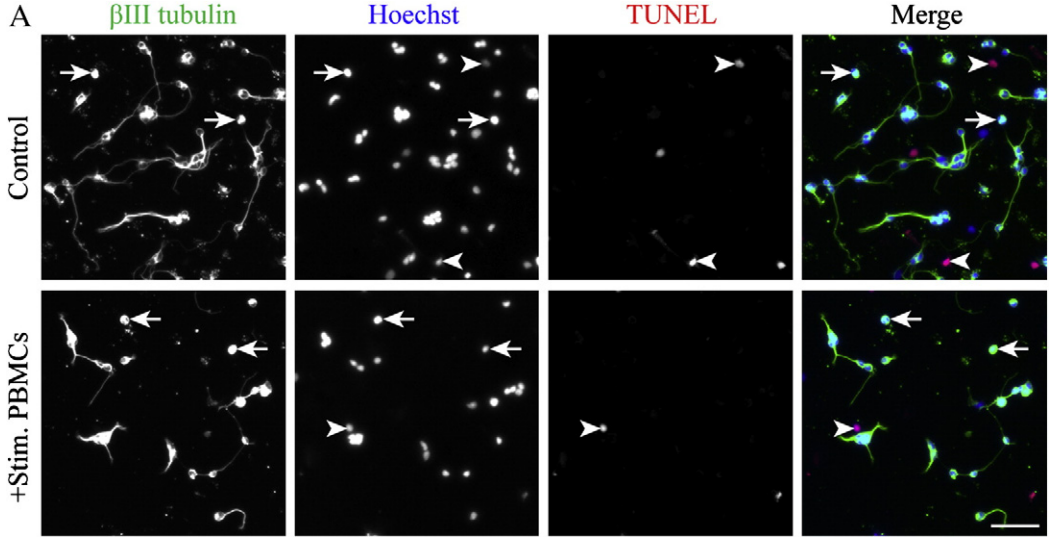

Fig. 2. PBMCs do not cause neuronal apoptosis. Neurons were assayed for apoptosis using a TUNEL assay. Representative images of untreated neurons (Control) and neurons co-cultured with PMA/ionomycin stimulated human PBMCs (+Stim. PBMCs) are shown. Note that neurons without neurite outgrowth remain TUNEL negative (arrows). Some βIII-tubulin negative (non-neuronal) cells are TUNEL positive (arrowheads). Scale bar = 50 μm.

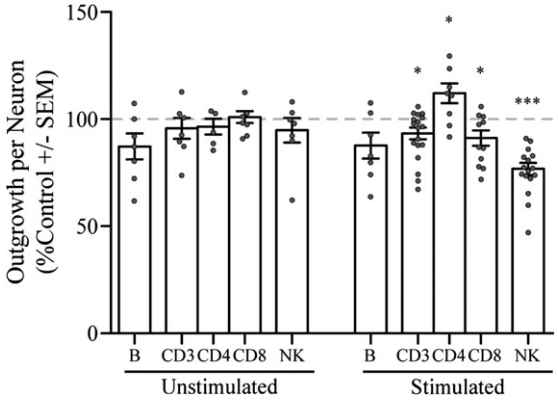

Fig. 3. Immune cell subsets exert distinct effects on neurite outgrowth. Quantification of human immune cell subset effects on neurite outgrowth/cell expressed as a percent of the outgrowth of neurons cultured without immune cells in each experiment ($n \geq 5$). Dots represent the results from individual experiments. The dashed line at 100% indicates the outgrowth of control neurons. The average extension of control neurons in these experiments was 23.0 +/− 0.930 μm/neuron. Statistical analysis by one-sample *t*-test compared to a hypothetical value of 100% indicates a significant effect of stimulated CD3+ T cells ($p = 0.0276$, 18), CD4+ T cells ($p = 0.0345$, $n = 5$), CD8+ T cells ($p = 0.0337$, $n = 7$), and NK cells ($p < 0.0001$, $n = 16$).

inhibitors (Table 2) of candidate pathways known to affect neurite outgrowth or degeneration for their ability to attenuate the growth inhibitory effect. Several of these inhibitors altered outgrowth of the neurons in the absence of immune cells (Fig. 5A). After normalizing the data to account for the baseline effects of the inhibitors, both inhibitors of the MAPK pathway (U0126 and PD098059), but no other

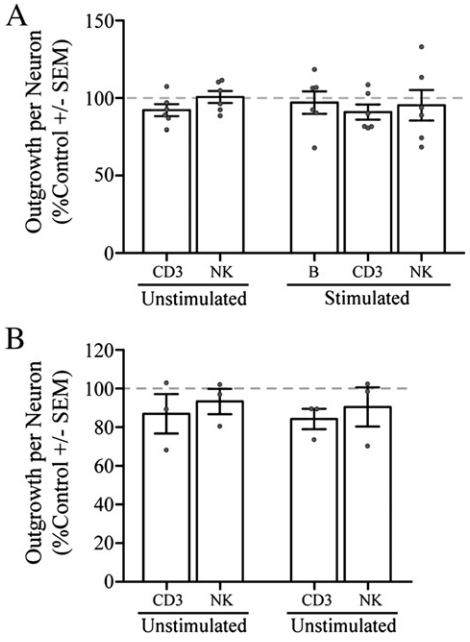

Fig. 4. Conditioned media from immune cell subsets fail to inhibit outgrowth. A) Quantification of neurite outgrowth from cerebellar neurons cultured in the presence of supernatants from immune cell subsets expressed as a percentage of the outgrowth in the presence of control medium collected from parallel wells cultured without immune cells ($n = 6$). Dots represent the results from individual experiments. The dashed line at 100% indicates the outgrowth of control neurons. Statistical analysis by one-sample *t*-test compared to a hypothetical value of 100% indicates no significant effect of any of the supernatants. B) Quantification of neurite outgrowth from cerebellar neurons cultured in transwells with either T cells of NK cells present in the upper chamber expressed as a percentage of the outgrowth in wells without immune cells in the upper chamber ($n = 3$). Dots represent the results from individual experiments. The dashed line at 100% indicates the outgrowth of control neurons. Statistical analysis by one-sample *t*-test compared to a hypothetical value of 100% indicates no significant effects of the immune cells.

Table 2
Pharmacological inhibitors.

Inhibitor	Target	Source	Final 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	MAPK	Sigma-Aldrich Canada	50 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

pharmacological inhibitors, significantly attenuated the NK cell inhibitory effect on neurite extension (Fig. 5B).

In the co-culture system, both the neurons and the NK cells were exposed to the inhibitors and the reversal of the phenotype could be ascribed to a requirement for MAPK activity in one or both of the cell types. To determine if the neurons responded to NK cells through a MAPK-dependent mechanism, we performed western blots to assess the level of phosphorylation of p42 and p44 MAPK in neurons exposed to NK cells. Phosphorylation of p42 and p44 MAPK does not change in neurons co-cultured with NK cells (Fig. 6A) suggesting that U0126 and PD098059 may affect the ability of NK cells to express an inhibitory factor. Because activation of the NK cells was required in order for them to acquire their outgrowth inhibitory properties (Fig. 3; compare unstimulated NK to stimulated NK), we assessed whether MAPK activity was regulated by NK cell activation. Western blotting for MAPK phosphorylation in NK cells reveals a dramatic increase in phospho-p42 and -p44 in activated cells (Fig. 6B; (Yu et al., 2000)) suggesting that high MAPK activity may be required in the NK cells to render them inhibitory. To address this question directly, we treated NK cells with U0126 or PD098059 for 24 h prior to co-culture and assessed their ability to inhibit outgrowth. In these co-cultures, there is a trend towards attenuated inhibition by the treated NK cells that is not statistically significant. Complete reversal is not expected, however, since the inhibitors are withdrawn for the 24-hour co-culture period of the outgrowth assay and the NK cells recover their MAPK activity (data not shown) and likely also their inhibitory properties during this time. Together the data is consistent with a model in which MAPK activity is required for the production of an inhibitory activity in NK cells.

Discussion

We report that total PBMCs are able to significantly inhibit neurite outgrowth and that this inhibition requires their activation. This overall PBMC effect reflects heterogeneous functions of distinct immune cells subsets. CD4+ T cells significantly promote neurite extension while, in contrast, CD8+ T cells and NK cells inhibit outgrowth with the most robust effects exerted by NK cells. This inhibition can be overcome using inhibitors of MAPK.

Promotion of outgrowth by CD4+ T cells

The significant promotion of outgrowth by CD4+ T cells reported here is similar to that in a recent report demonstrating that Th1-polarized T cells can promote outgrowth of cortical neurons (Ishii et al., 2010). The authors suggest that the mechanism is semaphorin 4A- (Sema4A) dependent as T cells from Sema4A knockout mice show

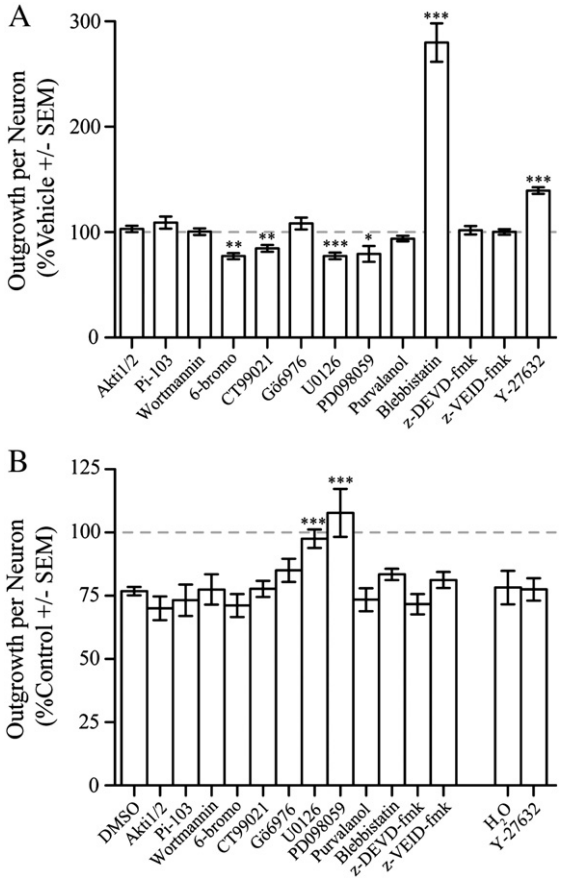

Fig. 5. NK cell effects on outgrowth are attenuated in the presence of MAPK inhibitors. A) Quantification of the effects of the inhibitors on outgrowth of the neurons alone expressed as a percentage of the outgrowth in the presence of the vehicle control ($n \geq 5$). The vehicle is DMSO for all inhibitors except for Y-27632 for which the vehicle is H_2O. The dashed line at 100% indicates the outgrowth of neurons in the presence of vehicle control. Statistical analysis by one-sample *t*-test compared to a hypothetical value of 100% indicates a significant effect of 6-bromo ($p = 0.0015$, $n = 5$), CT99021 ($p = 0.0059$, $n = 6$), U0126 ($p < 0.0001$, $n = 12$), PD098059 ($p = 0.0243$, $n = 9$), blebbistatin ($p < 0.0001$, $n = 11$), and Y-27632 ($p < 0.0001$, $n = 6$). B) Quantification of the effects of NK cells on outgrowth in the presence of inhibitors expressed as a percentage of the outgrowth in the presence of the inhibitor alone ($n \geq 5$). The dashed line at 100% indicates the outgrowth of control neurons. Statistical analysis by one-way repeated measures ANOVA with Dunnett's post-tests compared to the vehicle controls indicates a significant difference with the MAPK pathway inhibitors U0126 and PD098059 ($p < 0.001$ for both, $n = 12$ and $n = 9$, respectively). Please see Table 2 for a complete list of the targets of the inhibitors.

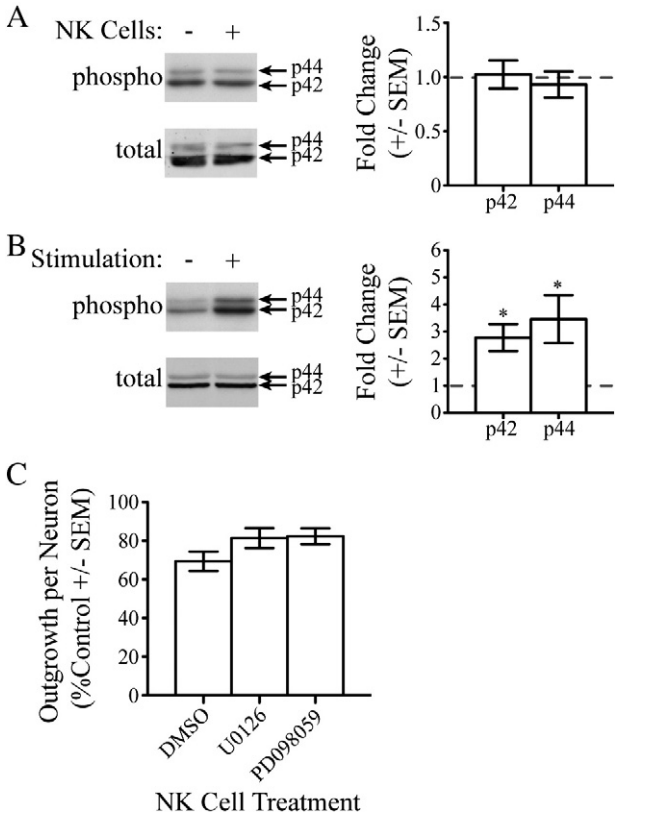

Fig. 6. MAPK activity is enhanced in NK cells that inhibit neurite outgrowth. A) Representative western blot of phosphorylated p44 and p42 MAPK in neurons exposed to NK cells. Densitometry was performed to measure the intensity of the phosphorylated p44 and p42 normalized to the intensity of the total p44 and p42 and the fold-change is expressed as a ratio of the values obtained for neurons in co-culture with NK cells compared to neurons alone. Statistical analysis by one-sample *t*-test compared to a hypothetical value of 1 shows no significant fold-change ($p = 0.8638$ for p42 and $p = 0.6080$ for p44; $n = 4$). B) Representative western blot of phosphorylated p44 and p42 MAPK in NK cells either unstimulated or stimulated for 3 days in culture. Densitometry was performed to measure the intensity of the phosphorylated p44 and p42 normalized to the intensity of the total p44 and p42 and the fold-change is expressed as a ratio of the values obtained for stimulated compared to unstimulated NK cells. Statistical analysis by one-sample *t*-test compared to a hypothetical value of 1 shows a significant fold-change for both proteins ($p = 0.0233$ for p42 and $p = 0.0496$ for p44; $n = 4$). C) Quantification of the neurite outgrowth effects of NK cells treated with MAPK inhibitors prior to co-culture expressed as a percentage of the neurons cultured alone ($n = 7$). Statistical analysis by one-way repeated measures ANOVA with Dunnett's post-tests compared to the DMSO control indicates no significant difference with NK cells treated with U0126 or PD098059 ($p = 0.0956$). However, a trend towards less neurite inhibition was noted.

a decreased ability to enhance outgrowth. However, given the important role of Sema4a in T cell activation and Th1/Th2 regulation (Kumanogoh et al., 2002, 2005), it is also possible that the failure of the knockout T cells to enhance neurite outgrowth is a result of a change in T cell phenotype rather than directly a lack of Sema4A expression by the T cells. These results suggest that the effects of T cells on outgrowth may be dependent on the context. Further, in CNS inflammation, the potential benefit of T cells will depend on the relative contributions of other infiltrating cells. When neurons are exposed to the total PBMC population, the effect on outgrowth is one of inhibition suggesting that either the functional properties of the CD4+ T cells are different in the presence of the other cells, or the potential growth promoting effects of the CD4+ T cells are overwhelmed by the inhibitory nature of the other cells present in the mixture. Thus, the overall effect on neuronal growth may depend on the number and type of the infiltrating cells. Nevertheless, these results suggest that there may be an endogenous immune cell pathway that could be harnessed to promote neuronal growth.

A sub-lethal neurite outgrowth inhibitory effect of NK and CD8+ T cells

Prior work has demonstrated that activated T cells are capable of efficiently killing human fetal neurons (Giuliani et al., 2003). However, a potential explanation for the substantial cell death reported in studies employing embryonic neurons is that neuronal apoptosis is a fundamental characteristic of the developing CNS (Boya and de la Rosa, 2005). We speculated that as neurons mature in vivo they become more resistant to programmed death and, as a consequence, less vulnerable to immune mediated killing. Since there is currently no protocol to culture adult human neurons, we examined effects of immune cells on neurite extension in a well-established post-natal rat cerebellar neuronal culture system. We observed that the neurite growth-inhibition mediated by exposure to immune cells was associated with minimal neuronal DNA damage. The ability of rat PBMCs to also robustly inhibit neurite outgrowth in our system indicates that this effect is not merely due to xenogeneic interactions, but rather represents a fundamental biological property of immune cells to mediate a form of sub-lethal

inhibition on neuronal growth in a syngeneic setting. Such an active inhibitory effect of immune cells on neurite extension could have substantial impact on the prospects of axonal repair in many contexts including MS lesions where injury is mainly to axons, with relative sparing of neuronal cell bodies (Grigoriadis et al., 2004).

In previous work, antigen/MHC-restricted T cells were shown to transect the axons of MHC I-expressing hippocampal neurons in the absence of detectable neuronal death (Medana et al., 2001). The murine hippocampal neurons used for that study were grown for 72 h prior to the addition of the immune cells. It is possible that, had the immune cells been added before neurons had formed extensions, outgrowth inhibition might have been detected as opposed to axonal transection. Both transection and inhibitory effects are applicable in the case of MS or acute injury, where axons are first damaged and later may be unable to regrow in part due to an immune cell-mediated inhibitory environment. It is also possible that following transection and with prolonged lack of regrowth, the cells eventually undergo apoptosis. In this case, cell death may not be a direct result of immune/neuron interaction and intervention early in the disease to reverse axonal outgrowth inhibition and promote reformation of connections may preserve the neurons.

Mechanism of action of leukocyte-dependent neurite outgrowth inhibition

Using the inhibitors PD098059 and U0126, we showed that MAPK activity was required for NK cells to exert an inhibitory effect in co-culture with neurons. We were unable to observe any change in MAPK activity in neurons exposed to NK cells by western blotting suggesting that MAPK signaling in neurons may not be critical for immune cell-dependent neurite outgrowth inhibition. However, it is still possible that localized or transient changes in MAPK activity that would be undetectable biochemically may be important for immune cell-dependent neurite outgrowth inhibition. MAPK phosphorylation was increased in NK cells when they were activated in culture correlating with their acquisition of an inhibitory activity. Blockade of MAPK in NK cells prior to co-culturing them with neurons failed to significantly reverse the inhibitory activity of these cells. However, the effect of the MAPK inhibitors does not last throughout the co-culture period (data not shown), thus it is also possible that production of a neurite outgrowth inhibitory molecule by NK cells is dependent on MAPK activity. Activation of MAPK is involved in cytolytic functions as well as induction and secretion of cytokines by NK cells (Vivier et al., 2004). Since we do not observe any increase in apoptosis in our neuronal cultures and since the supernatants of lymphoid lineage cells do not inhibit outgrowth, it is likely that the outgrowth inhibitory effect is mediated by one or more signaling molecule/s expressed on the NK cell surface.

Candidate molecules include several of the well-characterized ligands that inhibit neuronal outgrowth during development and/or after injury (Brose and Tessier-Lavigne, 2000; Culotti and Kolodkin, 1996; Grados-Munro and Fournier, 2003; Guthrie, 1999; Matsunaga and Chedotal, 2004; Tessier-Lavigne, 1995) as these have also been described in immune cells. These include ephrins, netrins, slits and semaphorins (Delaire et al., 1998; Wu et al., 2001; Yu et al., 2004). The signaling cascades of several of the inhibitory ligands converge on Rho-kinase (Alabed et al., 2006; Conrad et al., 2007; Gallo, 2006; Mimura et al., 2006; Wahl et al., 2000; Yukawa et al., 2005) but several have parallel signaling pathways including but not limited to targets of the pharmacological inhibitors used in our screen. Our screen does not therefore eliminate these ligands as potential candidates since they may be acting through alternate or multiple signaling pathways. Further studies into the molecular mechanism of immune cell-mediated neurite outgrowth inhibition will require attention to the possibility that more than one pathway and/or ligand is involved. Identification of these signals and the chemokines and/or cytokines responsible for upregulation of these factors will be necessary in order to fully understand this process.

Understanding regrowth in neuroinflammatory disease

Very little is known about how lost axonal connections may be repaired in neurological disease, but it is clear that clinical outcome will depend on the degree of damage inflicted and the mechanisms in place to repair that damage. As relates to MS, studies in which a single, targeted EAE lesion is created in the spinal cord, identified the formation of detour circuits around the lesion (Kerschensteiner et al., 2004). Axons appeared to be unable to grow through the lesion site, yet much of the myelin containing the classical myelin inhibitory molecules (Grados-Munro and Fournier, 2003; Nash et al., 2009; Yiu and He, 2006) was cleared from the lesion site and no new myelination was observed (Kerschensteiner et al., 2004). This, coupled with persistence of immune cells at the lesion site, raises the possibility that immune cells actively inhibited the growth of axons through the lesion, requiring the formation of detour circuits to enable recovery.

For functional recovery in vivo, formation of detour circuits or regrowth through lesion sites requires regrowth lengths in the millimeter range. The reductions in outgrowth length in our in vitro study are comparatively much smaller (about 10 μm/cell) because the outgrowth is measurable only after a short period (24 h). However, we expect that interactions between immune cells and neurons in vivo would occur over an extended period of time. For example, although the exact timing of immune cell infiltration in MS in difficult to assess, gadolinium-enhancing lesions visible by MRI can persist for weeks to months (Grossman et al., 1988; Miller et al., 1988). After acute spinal cord injury, inflammatory cells infiltrate within days but may persist up to a year (Fleming et al., 2006). Thus, in human disease, the prolonged presence of immune cells could lead to chronic long-term axon growth inhibition and therefore have a much larger impact in vivo than we were able to observe in vitro.

Our data suggests that some lymphocytes have the capacity to alter neuronal regrowth in the central nervous system and that the type of lymphocyte and context may determine the outcome. We found that both T cells and NK cells can modulate neurite outgrowth. The type of immune cell infiltrate varies in different CNS conditions (Amor et al., 2010; Rezai-Zadeh et al., 2009). T cells can infiltrate the CNS in MS, AD, PD, ALS and after acute injury. Although NK cells are relatively infrequent in MS lesions (Traugott and Raine, 1984), they do infiltrate the CNS in other conditions (for review see (Shi and Ransohoff, 2010)). These include pathogenic diseases of viral origin (Brehin et al., 2008; Mankowski et al., 2002; Thapa et al., 2007; Wuest and Carr, 2008) and other infectious origin (Hayashi et al., 2009) as well as trauma (Bona et al., 1999; Holmin et al., 1995). Thus, in a variety of medical conditions, lymphocytes can be in close contact with axons and therefore have the opportunity to modulate axon regrowth. The molecular pathways that mediate these neuronal growth modulatory effects remain to be elucidated, but these results may aid in the identification of a therapeutic target to promote repair within inflammatory CNS lesions.

Experimental methods

Preparation of immune cells and conditioned medium

Peripheral blood was collected by venous phlebotomy from healthy adult volunteers, recruited at the Montreal Neurological Institute following Institutional Review Board approved informed consent. Blood from adult Sprague–Dawley rats was collected by cardiac puncture following an animal use protocol approved by the Montreal Neurological Institute animal care committee according to Canadian Council on Animal Care guidelines. Human and rat PBMCs were separated by Ficoll-Paque density centrifugation and cultured as previously

described (Alter et al., 2003). The total PBMC population comprised essentially 50–70% CD3+ T cells, 10–20% CD19+ B cells, 5–10% CD56+ NK cells and 10–20% CD14+ monocytes. Total PBMCs were either left unstimulated or activated by adding phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich Canada, Oakville, ON) (P/I) at final concentrations of 0.08 μg/mL PMA and 1 μg/mL ionomycin. Human immune cell subsets were isolated from PBMCs using MACS® cell separation (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. T and B cells were isolated by positive selection using CD3 microbeads and CD19 microbeads, respectively. NK cells were isolated using the NK isolation kit. For T cell subsets, CD4+ and CD8+ T cells were isolated by a two-step MACS procedure — first by negative selection using the CD4 and CD8 cell isolation kits then by positive selection with CD4 and CD8 microbeads, respectively. Cell subset purity was greater than 90% as assessed by flow cytometry. Cell subset specific activations were as follows: plate bound anti-CD3 (UCHT-1) and anti-CD28 (CD28.2) for T cells (both at 0.5 μg/ml; Ebioscience, San Diego, CA), B cell receptor cross-linking antibody and irradiated L cells expressing CD40 ligand for B cells (Duddy et al., 2004) and IL-2 and IL-15 for NK cells (Darlington et al., 2008). Cells were cultured in Ultraculture serum-free medium (Cedarlane Laboratories, Burlington, ON) containing 1% penicillin–streptomycin (Invitrogen, Burlington, ON) for 3 days prior to collection of conditioned medium and cells for addition to neuronal cultures. Cells and conditioned medium were used only when the proportion of dead cells was less than 15% as assessed by trypan blue staining. Control conditioned medium was collected from wells lacking immune cells cultured in parallel under the same conditions. For experiments with treatment of NK cells with MAPK inhibitors prior to co-culture, the inhibitors were added for the last 24 h of the 3-day culture period at the concentrations indicated in Table 2.

Outgrowth assays

Total cerebellar neurons from P8 Sprague–Dawley rat pups (Charles River Canada, Montreal, QC) were prepared as described previously (Alabed et al., 2006) and plated at a density of 1100 cells/mm^2 on 0.01% Poly-L-Lysine (Sigma-Aldrich) coated 96 well plates in 1% N2 supplement (Invitrogen), 1% penicillin–streptomycin, 0.08 μg/ml tri-iodothyronine (Sigma-Aldrich Canada), 0.1 μg/ml L-thyroxine (Sigma-Aldrich Canada) in DMEM (Invitrogen). This yields cultures with 80–90% βIII-tubulin positive cells. Rat cortical and hippocampal neurons were prepared from embryonic day 17–18 rat brains. The cortices and hippocampi were dissected, dissociated with trypsin and mechanical trituration, and the cells were cultured on Poly-L-Lysine coated 96 well culture dishes in Neurobasal medium supplemented with 2% B27, 1% N2, 1% penicillin–streptomycin and 1% glutamine.

Neurons were cultured for 1 h prior to the addition of immune cells or conditioned medium supplemented with 1% N2 supplement, 0.08 μg/ml tri-iodothyronine, and 0.1 μg/ml L-thyroxine. Immune cells were collected by centrifugation, resuspended in cerebellar neuron medium and added to neuronal cultures at an immune cell to neuron ratio of 2:1. This ratio was chosen to try to encourage immune cell–neuron contacts without necessarily conditioning the media with an abundance of immune cell-secreted products. It is possible to estimate a plausible ratio of immune cells to axons in MS during an acute attack and for Parkinson's disease from data in the literature. The infiltrating T cell density in an active MS lesion is typically between 100 and 250 cells per mm^2 (Brück, 2005; Hauser et al., 1986; Marik et al., 2007; Traugott et al., 1983; Vercellino et al., 2009) and lesion diameter ranges from 1 mm to 30 mm but most lesions are between 2 and 8 mm (Wang et al., 1997). An axon passing through a 5 mm square section of active lesion would therefore potentially be in contact with 80 T cells (the square root of the product of the area of the lesion times the infiltrating cell density). In Parkinson's disease, there are far fewer infiltrating cells (0.4–3.6 CD8+ and 0.05–0.33 CD4+ cell/mm^2; (Brochard et al., 2009)) and these are not localized to focal lesions. Using the same formula, a 5 mm segment of axon could contact 3–9 CD8+ T cells and 1–2 CD4+ T cells. In the co-cultures used in the current study, immune cells are not attached to the substrate and can therefore make multiple contacts with multiple neurons during the course of the 24-hour culture period. Thus it is difficult to translate the ratio used in the co-culture paradigm to the in vivo state. Nevertheless, the occurrence of 5–80 contacts between immune cells and neurons during the co-culture period is not unreasonable (see Supplementary Videos for examples of immune cell neurons interactions in the co-cultures).

For experiments with pharmacological inhibitors, the inhibitors were added just prior to addition of immune cells at the concentrations listed in Table 2. Following 24 h of co-culture, neuronal cultures were fixed with 4% paraformaldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA) and stained with anti-βIII tubulin antibody (Covance, Berkeley, CA) to reveal the neuronal cell bodies and neurites and Hoescht 33342 dye (Sigma-Aldrich Canada) to reveal the nuclei. A 24-hour growth period is sufficient to allow reliable quantification of outgrowth but short enough to avoid the complex networks that form with longer-term cultures. The cultures were imaged using an ImageXpress micro instrument and 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 neuronal cells.

Evaluation of apoptosis by TUNEL assay and cleaved caspase-3 immunostaining

Free 3′ – OH ends of broken DNA strands were labeled with a TMR Red fluorescent marker using a commercially available In Situ Cell Death Detection Kit (Roche Applied Science, Laval, QC) according to the manufacturer's protocol. For cleaved caspase-3 assessment, an antibody that detects only the cleaved portion of caspase-3 (Cell Signaling Technology) was used. The cultures were counterstained with anti-βIII-tubulin and Hoechst 33342 and imaged using an ImageXpress micro instrument. The percentage of TUNEL positive or cleaved caspase-3 positive neurons was determined using the MultiWavelength Cell Scoring module of MetaXpress.

Assessment of MAPK phosphorylation

Neurons were isolated from NK cells in the co-culture by thoroughly washing off the non-adherent NK cells with ice-cold PBS three times. NK cells were washed by two sequential centrifugations and ice-cold PBS exchanges. Adherent cells or cell pellets were lysed in 50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100 containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (1 mM Na_3VO_4, 5 mM NaF). Lysates were analyzed by SDS–PAGE and immunoblotting with antibodies directed against phosphorylated (Thr202/Tyr204 in p44; Thr185/Tyr187 in p42) and total p42 and p44 MAPK (Cell Signaling Technology, Danvers, MA). Densitometry was performed using Adobe Photoshop to obtain the mean intensity of the bands.

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. 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 $p<0.05$.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2011.09.004.

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