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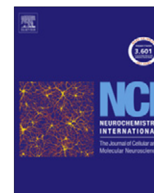

Detailed analysis of inflammatory and neuromodulatory cytokine secretion from human NT2 astrocytes using multiplex bead array

Kristina Burkert [a,c,1], Kiebashne Moodley [b,1], Catherine E. Angel [a,c], Anna Brooks [a,c], E. Scott Graham [b,*]

[a] School of Biological Sciences, Faculty of Science, University of Auckland, New Zealand
[b] Centre for Brain Research, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, New Zealand
[c] Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland 1142, New Zealand

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ABSTRACT

Astrocytes are a very important cell type in the brain fulfilling roles in both neuroimmunology and neurotransmission. We have conducted the most comprehensive analysis of secreted cytokines conducted to date (astrocytes of any source) to determine whether astrocytes derived from the human Ntera2 (NT2) cell-line are a good model of human primary astrocytes. We have compared the secretion of cytokines from NT2 astrocytes with those produced in astrocyte enriched human brain cultures and additional cytokines implicated in brain injury or known to be expressed in the human brain. The concentration of cytokines was measured in astrocyte conditioned media using multiplex bead array (MBA), where 18 cytokines were measured simultaneously. Resting NT2 astrocytes produced low levels (~1–30 pg/ml) of MIP1α, IL-6 and GM-CSF and higher levels of MCP-1, IP-10 and IL-8 (1–11 ng/ml) under non-inflammatory conditions. All of these in addition to IL-1β, TNFα, and IL-13, were increased by pro-inflammatory activation (TNFα or IL-1β stimulation). In contrast, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, LTα, and IFNγ were not detected in astrocyte conditioned media under any of the culture conditions tested. NT2 astrocytes were unresponsive to IL-2 and the adenyl cyclase agonist, forskolin. Interestingly, IFNγ stimulation selectively increased IP-10 secretion only. As astrocytes stimulated with IL-1β or TNFα produced several chemokines in the ng/ml range, we next assessed the chemoattractant properties of these cells. Conditioned media from TNFα-stimulated astrocytes significantly chemoattracted leukocytes from human blood. This study provides the most comprehensive analysis of cytokine production by human astrocytes thus far, and shows that NT2 astrocytes are highly responsive to pro-inflammatory mediators including TNFα and IL-1β, producing cytokines and chemokines capable of attracting leukocytes from human blood. We conclude that in the absence of adult human primary astrocytes that NT2-astrocytes may provide a valuable alternative to study the immunological behaviour of human astrocytes.

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1. Introduction

In the last decade, our understanding of the multifaceted functions of the astrocyte has increased exponentially. Astrocytes are very important for providing neurological support, maintaining integrity of the blood brain barrier (BBB) and for providing an important arm of the immune system within the brain. With regard to the later astrocytes have the capacity to produce a variety of inflammatory cytokines and chemokines (Aloisi et al., 1992; Choi et al., 2002; Janabi et al., 1999; Sharma et al., 2007), as well as cytokines that are likely involved in homeostasis of the BBB and neurons (Bsibsi et al., 2006; Meeuwsen et al., 2003). During the early stages of neuroinflammation astrocytes are thought to be involved in reparative processes. However, as producers of potent inflammatory mediators and chemokines known to recruit peripheral leukocytes into the inflamed CNS (Meeuwsen et al., 2003; Williams et al., 2009b), they also have the potential to contribute to some of the inflammation mediated damage that occurs in some neuroinflammatory diseases (e.g. stroke, MS). Therefore their role is very much a double-edge sword where the compromise of the local brain-barrier environment likely plays a significant part in the overall clinical outcome.

With better understanding of astrocyte function comes increased demand for primary astrocytes from human brain tissue to investigate functional modalities. Primary adult human astrocytes are the absolute gold standard for studying astrocyte immunology and communication with neuronal cells. However, there are

Abbreviations: NT2, Ntera2; CBA, cytometric bead array; GMB, glioblastoma multiforme; MFI, mean fluorescent intensity.

* Corresponding author. Tel.: +64 9 3737599x86947.
E-mail address: s.graham@aukcland.ac.nz (E.S. Graham).

[1] These authors contributed equally to this work.

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several inherent issues, which significantly limiting the nature and extent of research capable by the wider neuroscience research community (i.e. availability and yield). Access to human brain tissue to obtain primary cultures is extremely limited for many researchers. Moreover, for those fortunate to have access to human brain tissue (post-mortem or biopsy), the yields of primary astrocytes can be very low (especially from post-mortem tissue), which significantly limits the ability to conduct extensive functional studies. Another important consideration, is that astrocyte cultures will undoubtedly contain other brain cells, such as microglia, fibroblast-like cells and endothelial cells. These cell types all produce a repertoire of cytokines, therefore it is important to consider the source of cytokines measured in primary cultures, especially where gene-array studies or secretion is being measured, as these do not take into consideration to cellular source. Therefore, there is a great need for human astrocyte cell-lines that model the major functional characteristics of primary astrocytes. This is required for investigating astrocyte functions in the healthy brain (e.g. glutamate levels, neuronal homeostasis, BBB integrity, etc.) and also for investigating functions in various disease states (neuroinflammation; e.g. acute brain injury, multiple sclerosis, stroke, degeneration, or astroglial tumours).

The Ntera2/D1 cell line is relatively well characterised regarding differentiation of various neuronal phenotypes from the neuroepithelial precursors (Daadi et al., 2001; Hartley et al., 1999a; Hurlbert et al., 1999; Pleasure and Lee, 1993; Pleasure et al., 1992; Saporta et al., 2001). Differentiation of astrocytes from NT2 precursors is a more recent development (Sandhu et al., 2002a) and the immunological phenotype of NT2 astrocytes has not been investigated in detail (Sandhu et al., 2002a). Therefore, the aim of this research was to conduct an extensive analysis of cytokine production by NT2 astrocytes and determine whether NT2 astrocytes secrete leukocyte-recruiting chemokines. In this study, we have focused on cytokines detected in primary human astrocyte cultures to directly validate the immunology phenotype of the NT2 astrocytes. In addition, we have measured the production of other cytokines that have an important bearing in neurological diseases (summarised in Table 1). These data indicate that the cytokine profile of NT2-astrocytes is very similar to that of primary human astrocytes we conclude, NT2 astrocytes may provide a valuable tool for studying neuroinflammation mediated by astrocytes, especially where access to human primary cells is not possible.

2. Methods

2.1. Differentiation of NT2 astrocytes

All media, serum and antibiotics were purchased from Invitrogen. Cytokines used for stimulating the astrocytes were purchased from PeproTech (New Jersey, US). The Ntera2/D1 cell line was purchased from ATCC. Astrocytes were produced using a 10–11 week differentiation protocol (Goodfellow et al., 2011; Lim et al., 2007; Unsworth et al., 2011). NT2 astrocytes were cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin (DF10). Precursor NT2 cells were seeded at ~2.5 million cells/T75 culture flask and allowed to settle for 24 h. The media was replaced with DF10 and a supplement of 10 μM retinoic acid (RA). The

Table 1
List of all the cytokines investigated in this study.

Cytokine	Aliases	Produced by human primary astrocytes	Produced by human astrocytic line	Rationale
IL-1β (#55879)	LAF, MCF	Yes (Aloisi et al., 1992; Choi et al., 2002)		Produced by human astrocytes or astrocytic cell line*
IL-2 (3558270)	TCGF		Yes (Choi et al., 2002)	As above
IL-4 (#558272)	BCGF	mRNA (Meeuwsen et al., 2003)	Yes (Choi et al., 2002)	As above
IL-6 (#558276)	IFN-β2, BCDF	Yes (Aloisi et al., 1992; Sharma et al., 2007)	Yes (Spooren et al., 2011)	As above
IL-8 (#558277)	CXCL8	Yes (Aloisi et al., 1992; Janabi et al., 1999)	Yes (Lu et al., 2005)	As above
IL-10 (#558274)	B-TCGF	Yes (Bsibsi et al., 2006)		As above
IL-12 (#558283)	NKSF, CTL-SF	Yes (Bsibsi et al., 2006)		As above
GM-CSF (#558335)	CSF-2,	Yes (Aloisi et al., 1992)		As above
MCP-1 (#558287)	CCL2	Yes (Sharma et al., 2007; Sheng et al., 2005)		As above
MIP-1α (#558325)	CCL3	Yes (Janabi et al., 1999)		As above
IFNγ (#558335)	Type II interferon		Yes (Choi et al., 2002)	As above
IP-10 (#558280)	CXCL10	Yes (Sharma et al., 2007; Sheng et al., 2005)	Yes (Williams et al., 2009b)	As above
TNF-α (#558273)		mRNA (Bsibsi et al., 2006)	Yes (Choi et al., 2002)	As above
Others				
IL-5 (#558278)	EDF	mRNA (Bsibsi et al., 2006)		IL-5 receptors expressed by human microglia (Lee et al., 2002) IL-5 mRNA expressed by neurons and rat astrocytes (Lins and Borojevic, 2001)
IL-7 (#558334)	LP-1			Selectively expressed by some neurons and endothelial cells
IL-13 (#558450)	NC30			IL-13R highly expressed in gliomas (Kawakami et al., 2001; Rahaman et al., 2005)
Fas Ligand (#558330)	CD95L, TNFSF6	mRNA (Meeuwsen et al., 2003) mRNA (Bsibsi et al., 2006)		Fas Ligand kills receptor bearing cells. Important in tumour cell death
LT-α (#560083)	TNF-β			Potent mediator of inflammatory responses. Cytotoxic to tumour cells

The catalogue number (BD Biosciences CBA flex-set) for each cytokine is detailed beside the name in the first column. Detailed are their common aliases, and rationale for inclusion in the study including which cytokines have been shown previously to be secreted from astrocyte-enriched cultures from the human brain.

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